

# IDENTIFICATION OF GENES INVOLVED IN ALZHEIMER'S DISEASE USING DROSOPHILA MELANOGASTER



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## BACKGROUND OF THE INVENTION

Alzheimer's disease (AD) is a neurological disorder resulting in the degeneration and eventual death of neurons in brain centers controlling memory, cognition and behavior. The hallmark of the disease is the formation of insoluble amyloid deposits (senile plaques), the major component of which is the 40-42 amino acid amyloid beta (A $\beta$ ) peptide, a proteolytic product of the amyloid precursor protein (APP). These plaques are widely believed to be the major causative agents leading to the degeneration and death of neuronal cells.

The three major known genes associated with inheritance of familial Alzheimer's disease (FAD) in humans are the transmembrane receptor *amyloid precursor protein* (APP) and the two *presenilin* (PS1 and PS2) genes. Missense mutations in these genes result in the increased production of the A $\beta$  peptide, underscoring the importance of this peptide in contributing to the disease state. APP is cleaved at two sites, beta and gamma, to release a 40-42 amino acid peptide, A $\beta$  (reviewed in Mills, J. and Reiner, P.B. (1999) *J. Neurochem* 72: 443-460). Missense mutations in APP near the gamma site (Goate, A. et al., (1991). *Nature* 349: 704-706.), where the C-terminal end of the peptide is cleaved, result in production of more A $\beta$  42, by altering the 40/42 ratio (Suzuki, N., et al. (1994). *Science* 264: 1336-1340). Mutations around the beta site result in more overall production of both forms (Mullan, M., et al. (1992). *Nat. Genet.* 1: 345-347.); Citron, M. et al. (1995). *Neuron* 14: 661-670).

The presenilins are multiple pass transmembrane proteins, the functions of which are currently a matter of debate. Missense mutations in presenilins increase the release of the A $\beta$  42 form (Borchelt, D.R., et al. (1996). *Neuron* 17: 1005-1013); Citron, M., et al. (1997). *Nat. Med.* 3: 67-72; Murayama, O. et al. (1999). *Neurosci. Lett.* 265: 61-63) and

account for the majority of FAD cases (Sherrington, R., et al.(1995). *Nature* 375: 754-760).

Many studies have examined the roles of both the soluble and insoluble (aggregated) forms of A $\beta$  and it is widely believed that the aggregated form of the peptide is responsible for the observed toxic effects (Pike, C.J., et al. (1993). *J. Neurosci.* 13: 1676-1687; Lorenzo, A. and Yankner, B.A. (1994). *Proc. Natl. Acad. Sci. USA* 91: 12243-12247; Giovannelli, L., et al (1998). *Neurosci.* 87: 349-357). There are a number of mechanisms that contribute to A $\beta$ -induced death of neurons, including the disruption of intracellular calcium levels (for reviews, see Fraser, S.P., et al. (1997). *Trends Neurosci.* 20: 67-72; Mattson, M.P. (1997). *Physiol. Rev.* 77: 1081-1132; Coughlan, C.M. and Breen, K.C. (2000). *Pharmacol. and Ther.* 86: 111-144), the induction of an inflammatory response caused by activation of microglial cells (reviewed in Coughlan, C.M. and Breen, K.C. (2000). *Pharmacol. and Ther.* 86: 111-144) and the marked degeneration and/or disruption of the basal-forebrain cholinergic system, which is involved in learning and memory (reviewed in Hellström-Lindahl and Court, 2000, *Behav. Brain Res.* 113 (1-2): 159-68). Thus, it is clear that the deleterious effects of A $\beta$  overproduction and its contribution to AD are numerous and complex.

Although a great amount of research has been dedicated to the study of Alzheimer's Disease and its general pathology, the genetic analysis of human neurodegenerative disorders is limited. As a result, the events that trigger the accumulation of beta amyloid, as well as the precise role of genes such as *APP* and others suspected to play a part in Alzheimer's Disease, is poorly understood.

Numerous contributions to the establishment of the central role of A $\beta$  in the manifestation and progression of AD have come from studies in model systems. Transgenic mice expressing either wild type or mutant forms of APP exhibit AD pathology, in many cases developing amyloid plaques in an age-dependent fashion and in some cases displaying altered behavior and cognition (for reviews, see Price, D.L., et al (1998). *Annu. Rev. Genet.* 32: 461-493; van Leuven, F. (2000). *Progress in Neurobiol.*

61: 305-312). Transgenic mice expressing only the A $\beta$  42 peptide exhibit extensive neuronal degeneration in brain regions normally affected in AD, and 50% die at 12 months of age (LaFerla, F.M. et al. (1995). *Nature Genet.* 9: 21-30). The neural cells in these mice eventually apoptose, followed by astrogliosis and spongiosis. This demonstrates that A $\beta$  42 expression is toxic in vivo, and results in neuronal degeneration and apoptosis.

The use of *Drosophila* as a model organism has proven to be an important tool in the elucidation of human neurodegenerative disease pathways (reviewed in Fortini, M and Bonini, N. (2000). *Trends Genet.* 16: 161-167), as the *Drosophila* genome contains many relevant human orthologs that are extremely well conserved in function (Rubin, G. M., et al. (2000). *Science* 287: 2204-15). For example, *Drosophila melanogaster* carries a gene that is homologous to human *APP* which is involved in nervous system function. The gene, *APP-like (Appl)*, is approximately 40% identical to the neurogenic isoform (695) of the human *APP* gene over three large domains (Rosen et al., PNAS USA 86:2478-2482 (1988)) and, like human *APP695*, is exclusively expressed in the nervous system. Flies deficient for the *Appl* gene show behavioral defects which can be rescued by the human *APP* gene, suggesting that the two genes have similar functions in the two organisms (Luo et al., *Neuron* 9:595-605 (1992)).

In addition, *Drosophila* models of polyglutamine repeat diseases (Jackson, G.R., et al (1998). *Neuron* 21: 633-642; Kazemi-Esfarjani, P. and Benzer, S. (2000). *Science* 287: 1837-1840; Fernandez-Funez et al. (2000) *Nature* 408 (6808):101-6, and Parkinson's disease (Feany, M.B. and Bender, W.W. (2000). *Nature* 404: 394-398) closely mimic the disease state in humans, both at the cellular as well as the physiological level and have been used successfully to identify other genes that play a role in these diseases. Thus, the power of *Drosophila* as a model system is demonstrated in the ability to represent the disease state and to perform large scale genetic screens.

This invention generally relates to a method to identify compounds and genes acting on the APP pathway in transgenic *Drosophila melanogaster* ectopically expressing

genes related to AD. Expression of these transgenes can induce visible phenotypes and it is contemplated herein that genetic screens disclosed herein may be used to identify genes involved in the APP pathway by the identification of mutations that modify the induced visible phenotypes. The genes affected by these mutations will be called herein "genetic modifiers". It is contemplated herein that human homologs of genetic modifiers thus identified would be useful targets for development of therapeutics to treat conditions associated with abnormalities in the APP pathway, including, but not limited to, the development of Alzheimer Disease (AD) therapeutics. It is also contemplated herein that some of these human homologs might be occurring on an area of human chromosome 10, shown to be linked to Alzheimer's disease (Bertram *et al.*, Ertekin-Taner *et al.*, Myers *et al.*, *Science* 290, 2302-2305, 2000). Such human homologs might have the potential to be genetically linked to AD and serve as markers for AD or as targets for the development of therapeutics to treat conditions associated with abnormalities in the APP pathway, including, but not limited to, the development of Alzheimer Disease (AD) therapeutics. Such human homologs might also be acting in cellular pathways involving genes linked to AD and these human homologs might be used to identify the genes in these pathways.

## SUMMARY OF THE INVENTION

The present invention pertains to a transgenic fly whose genome comprises a DNA sequence encoding a polypeptide comprising the Abeta portion of human *APP* wherein said DNA sequence encodes Abeta40 (SEQ ID: NO 1) or Abeta42 (SEQ ID: NO 2), fused to a signal sequence, said DNA sequence operably linked to a tissue-specific expression control sequence; and expressing said DNA sequence, wherein expression of said DNA sequence results in said fly displaying an altered phenotype. In one particular embodiment, the DNA sequence encodes Abeta42, the tissue specific expression control sequence comprises the eye-specific promoter GMR and expression of the DNA sequence results in an altered phenotype referred to as the "rough eye" phenotype.

In a further aspect, the invention pertains to a transgenic fly whose genome comprises a DNA sequence encoding a polypeptide comprising the wild type C99 portion of human *APP* (SEQ. ID NO:3) or C99 portion of human *APP* with the London Mutation (SEQ ID NO: 4) fused to a signal sequence, said DNA sequence operably  
5 linked to a tissue-specific expression control sequence; and expressing said DNA sequence, wherein expression of said DNA sequence results in said fly displaying an altered phenotype. In one embodiment, the DNA sequence encodes the wild type C99, the tissue-specific expression control sequence is the UAS control element, which is  
10 activated by Gal4 protein produced in the brain by the 7B-Gal4 transgene and expression of the DNA sequence results in an altered phenotype characterized by a locomotory defect. In another particular embodiment, the DNA sequence encodes either the wild type C99 or the C99 portion of human *APP* with the London Mutation, the tissue-specific expression control sequence is UAS control element activated by Gal4 protein produced by the apterous-Gal4 transgene and expression of the DNA sequence results in an altered  
15 phenotype referred to as the "concave wing" phenotype.

In a further aspect, the invention pertains to a method to identify genetic modifiers of the APP pathway, said method comprising providing a transgenic fly whose genome comprises a DNA sequence encoding a polypeptide comprising the Abeta portion of  
20 human *APP* wherein said DNA sequence encodes Abeta40 (SEQ ID NO: 1) or Abeta42 (SEQ ID NO: 2), fused to a signal sequence, said DNA sequence operably linked to a tissue-specific expression control sequence; and expressing said DNA sequence, wherein expression of said DNA sequence results in said fly displaying an altered phenotype; crossing said transgenic fly with a fly containing a mutation in a known or predicted  
25 gene; and screening progeny of said crosses for flies that carry said DNA sequence and said mutation and display modified expression of the transgenic phenotype as compared to controls. In one embodiment, the DNA sequence encodes Abeta42, the tissue specific expression control sequence comprises the eye-specific promoter GMR and expression of said DNA sequence results in said fly displaying an altered phenotype referred to as the  
30 "rough eye" phenotype.

In a further aspect, the invention pertains to a method to identify genetic modifiers of the APP pathway, said method comprising: providing a transgenic fly whose genome comprises a DNA sequence encoding a polypeptide comprising the wild type C99 portion of human *APP* (SEQ. ID NO:3) or C99 portion of human *APP* with the London Mutation (SEQ ID NO: 4) fused to a signal sequence, said DNA sequence operably linked to a tissue-specific expression control sequence; and expressing said DNA sequence, wherein expression of said DNA sequence results in said fly displaying an altered phenotype; crossing said transgenic fly with a fly containing a mutation in a known or predicted gene; and, screening progeny of said crosses for flies that carry said DNA sequence and said mutation and display modified expression of the transgenic phenotype as compared to controls. In one embodiment, the DNA sequence encodes the wild type C99, the tissue-specific expression control sequence is the UAS control element, activated by Gal4 protein produced in the brain by the 7B-Gal4 transgene and expression of said DNA sequence results in said fly displaying an altered phenotype characterized by a locomotory defect. In another embodiment, the DNA sequence encodes either the wild type C99 or the C99 portion of human *APP* with the London Mutation, the tissue-specific expression control sequence is UAS control element activated by Gal4 protein produced by the apterous-Gal4 transgene and expression of said DNA sequence results in said fly displaying an altered phenotype referred to as the "concave wing" phenotype.

A further aspect of the invention pertains to a method to identify compounds that act on gene products involved in the APP pathway by assaying for compounds that can modify the phenotypes induced by expression of Abeta, said method comprising: providing a transgenic fly whose genome comprises a DNA sequence encoding a polypeptide comprising the Abeta portion of human *APP* wherein said DNA sequence encodes Abeta40 (SEQ ID NO: 1) or Abeta42 (SEQ ID NO: 2), fused to a signal sequence, said DNA sequence operably linked to a tissue-specific expression control sequence; and expressing said DNA sequence, wherein expression of said DNA sequence results in said fly displaying an altered phenotype; administering to said fly a candidate compound; and, assaying for changes in the phenotype of said fly as compared to the phenotype of a similar transgenic fly not administered the candidate compound. In

one embodiment, the DNA sequence encodes Abeta42, the tissue specific expression control sequence is the eye-specific promoter GMR and expression of said DNA sequence results in said fly displaying an altered phenotype referred to as the "rough eye" phenotype.

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Yet another aspect of the invention pertains to a method to identify compounds that act on gene products involved in the APP pathway by assaying for compounds that can modify the phenotypes induced by expression of C99, said method comprising: providing a transgenic fly whose genome comprises a DNA sequence encoding a polypeptide comprising the wild type C99 portion of human *APP* (SEQ. ID NO:3) or C99 portion of human *APP* with the London Mutation (SEQ ID NO: 4) fused to a signal sequence, said DNA sequence operably linked to a tissue-specific expression control sequence; and expressing said DNA sequence, wherein expression of said DNA sequence results in said fly displaying an altered phenotype; administering to said fly a candidate compound; and, assaying for changes in the phenotype of said fly as compared to the phenotype of a similar transgenic fly not administered the candidate compound. In one embodiment, the DNA sequence encodes wild type C99, the tissue-specific expression control sequence is the UAS control element activated by Gal4 protein produced in the brain by the 7B-Gal4 transgene and expression of said DNA sequence results in said fly displaying a phenotype characterized as a locomotory defect. In another embodiment, the DNA sequence encodes either wild type C99 or the C99 portion of human *APP* with the London Mutation, the tissue-specific expression control sequence is UAS control element activated by Gal4 protein produced by the apterous-Gal4 transgene and expression of said DNA sequence results in said fly displaying an altered phenotype referred to as the "concave wing" phenotype.

In yet another aspect, the invention pertains to a method for identifying genes involved in the onset or progression of conditions associated with abnormal regulation of the APP pathway, including but not limited to Alzheimer's Disease, and whose protein products might serve as potential markers for Alzheimer's Disease, said method

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comprising identifying the human homologs of fly genes that have been identified as genetic modifiers according to the methods of the present invention.

In yet another aspect, the invention pertains to a method for identifying genes involved in the onset or progression of conditions associated with abnormal regulation of the APP pathway, including but not limited to Alzheimer's Disease, and whose protein products might serve as potential markers for Alzheimer's Disease, said method comprising identifying human homologs of fly genetic modifier genes that are located close to the area of human chromosome 10 that is shown to have genetic linkage to Alzheimer's Disease.

In yet another aspect, the invention pertains to a method for identifying genes involved in the onset or progression of Alzheimer's Disease and whose protein products might serve as potential markers for AD, said method comprising identifying genes that are involved in the pathways regulated by the transcription factors encoded by the human sequences hCP50765 (SEQ ID NO. 35, encoded by the EGR2 gene), and hCP41313 (Seq ID NO 15 , SEQ ID NO17 or SEQ ID NO 53, encoded by the human homolog of the Drosophila nocA gene), which human sequences are located close to the area of human chromosome 10 that is shown to have genetic linkage to Alzheimer's Disease.

In yet another aspect, the invention pertains to a method for identifying compounds useful for the treatment, prevention or amelioration of pathological conditions associated with defects in the APP pathway, including but not limited Alzheimer's Disease, comprising administering candidate compounds to an in vitro or in vivo model of Alzheimer's Disease; and assaying for changes in expression of a genetic homolog of a genetic modifier, wherein altered expression of any one of said homologs compared to levels in a control to which a candidate compound has not been administered indicates a compound of potential therapeutic value.



The invention also pertains to a method for the treatment, prevention or amelioration of pathological conditions associated with defects in the APP pathway, including, but not limited to Alzheimer's Disease, comprising administering to a subject in need thereof a therapeutically effective amount of a compound that may inhibit or promote the function of any one or more of the polypeptide encoded by the human homologs of the genetic modifiers identified herein.

The invention also pertains to a method for the treatment, prevention or amelioration of pathological conditions associated with defects in the regulation of the APP pathway, including but not limited to Alzheimer's Disease, comprising administering to a subject in need thereof a therapeutically effective amount of a pharmaceutical composition comprising any one or more substances selected from the group consisting of: triple helix DNA, antisense oligonucleotides or ribozymes, all complementary to the appropriate sequence of a mRNA deriving from any one or more of the human homologs of genetic modifier genes identified according to the methods of the present invention.

The invention also pertains to a method for the treatment, prevention or amelioration of pathological conditions associated with defects in the regulation of the APP pathway, including but not limited to Alzheimer's Disease, comprising administering to a subject in need thereof a therapeutically effective amount of a pharmaceutical composition comprising double stranded RNA molecules directed to one or more of the human homologs of the genetic modifiers identified according to the methods of the present invention.

In a further aspect, the invention pertains to a method for the treatment, prevention or amelioration of pathological conditions associated with defects in the APP pathway, including but not limited to Alzheimer's Disease, comprising administering to a subject in need thereof a therapeutically effective amount of a pharmaceutical composition comprising an antibody or antibodies and/or fragments thereof directed to the polypeptide encoded by any one or more of the human homolog of the genetic modifiers identified according to the methods of the present invention .

In a further aspect, the invention also pertains to a method for the diagnosis of pathological conditions associated with abnormalities in the APP pathway in a subject, including but not limited to Alzheimer's Disease, which comprises measuring the mRNA level or the level or activity of the polypeptides encoded by any one or more of the human homologs of a genetic modifier in a biological sample from a subject, wherein an abnormal level relative to the level thereof in a control subject is diagnostic of said conditions.

In a still further aspect, the invention pertains to a kit comprising the components necessary to detect expression levels of polypeptides encoded by any one or more of the human homologs of a genetic modifier or fragments thereof or polynucleotides encoding any one or more of said polypeptides or fragments thereof, in a biological sample from a subject, such kits comprising antibodies that bind to said polypeptides or to said fragments thereof, or oligonucleotide probes that hybridize with said polynucleotides or to said fragments thereof and instructions for using said kit.

In yet another aspect, the invention pertains to a pharmaceutical composition comprising substances selected from the group consisting of: antisense, ribozyme, double stranded RNA or triple helix nucleic acids directed to any one or more of the human homologs of a genetic modifier or fragments thereof, polypeptides encoded by any one or more of the human homologs of a genetic modifiers or fragments thereof, polynucleotides encoding said polypeptides or fragments thereof, and antibodies that bind to said polypeptides or fragments thereof, in conjunction with a suitable pharmaceutical carrier, excipient or diluent, for the treatment of pathological conditions associated with abnormalities in the APP pathway, including but not limited to, Alzheimer's Disease.

The invention also pertains to a method for the treatment of pathological conditions associated with abnormalities in APP pathway including but not limited to, Alzheimer's Disease, comprising introducing nucleic acids encoding any one or more of the human homologs of a genetic modifier into one or more tissues of a subject in need thereof

resulting in that one or more proteins encoded by the nucleic acids are expressed and or secreted by cells within the tissue.

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## DETAILED DESCRIPTION OF THE INVENTION

All patent applications, patents, literature and website references cited herein are  
10 hereby incorporated by reference in their entirety.

In practicing the present invention, many conventional techniques in molecular biology and recombinant DNA are used. These techniques are well known and are explained in, for example, *Current Protocols in Molecular Biology, Volumes I, II, and*  
15 *III*, 1997 (F. M. Ausubel ed.); Sambrook et al., 1989, *Molecular Cloning: A Laboratory Manual, Second Edition*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.; *DNA Cloning: A Practical Approach, Volumes I and II*, 1985 (D. N. Glover ed.); *A Practical Guide to Molecular Cloning; the series, Methods in Enzymology* (Academic Press, Inc.); *Gene Transfer Vectors for Mammalian Cells*, 1987 (J. H. Miller and M. P.  
20 Calos eds., Cold Spring Harbor Laboratory); and *Methods in Enzymology Vol. 154 and Vol. 155* (Wu and Grossman, and Wu, eds., respectively). Well known *Drosophila* molecular genetics techniques can be found, for example, in Robert, D.B., *Drosophila, A Practical Approach* (IRL Press, Washington, D.C., 1986). Descriptions of flystocks can be found in the Flybase data base at <http://flybase.bio.indiana.edu> .

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A "transgenic" organism as used herein refers to an organism that has had extra genetic material inserted into its genome. As used herein, a "transgenic fly" includes embryonic, larval and adult forms of *Drosophila melanogaster* that contain a DNA sequence from the same or another organism randomly inserted into their genome.  
30 Although *Drosophila melanogaster* is preferred, it is contemplated that any fly of the genus *Drosophila* may be used in the present invention.

As used herein, "ectopic" expression of the transgene refers to expression of the transgene in a tissue or cell or at a specific developmental stage where it is not normally expressed.

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As used herein, "phenotype" refers to the observable physical or biochemical characteristics of an organism as determined by both genetic makeup and environmental influences.

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As used herein, a compound that may "inhibit or promote the function of any or more of the polypeptides encoded by the human homolog of a genetic modifier" includes compounds that may do so indirectly (via down stream effects) or directly, by binding to or otherwise interacting with the protein and includes, but is not limited to, antagonists or agonists of the protein.

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As used herein, a "stringent ortholog" is defined as meeting the following criteria: fly protein X has best match with human protein Y and fly protein X does not have a better match with another fly protein than with human protein Y and human protein Y has best match with fly protein X and human protein Y has no better match with another human protein than with fly protein X, wherein "X" and "Y" stand for any two fly and human proteins being compared.

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A "putative ortholog" is defined herein as meeting only the following two criteria: fly protein X has best match with human protein Y and human protein Y has best match with fly protein X, regardless of whether fly protein X had a better match with another fly protein and/or whether human protein Y had a better match with another human protein. As disclosed herein, all other human/fly protein matches are deemed "homologs".

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As used herein, the term "expression control sequence" refers to promoters and enhancers. The term "promoter" refers to DNA sequences which are recognized directly

or indirectly and bound by a DNA-dependent RNA polymerase during the initiation of transcription and includes enhancer elements. Enhancers used in the present invention include the UAS element which is activated by the yeast Gal4 transcriptional regulator.

5 The term "transcription factor" refers to any protein required to initiate or regulate transcription in eukaryotes. For example, the eye-specific promoter GMR is a binding site for the eye-specific transcription factor, GLASS (Moses, K and Rubin, *GM Genes Dev.* 5(4):583-93 (1991)).

10 As used herein, the term "Abeta" ( $A\beta$ ) refers to beta amyloid peptide which is a short (40-42 amino acid) peptide produced by proteolytic cleavage of APP by beta and gamma secretases. It is the primary component of amyloid depositions, the hallmark of AD and the cause of neuronal cell death and degeneration. Abeta peptide of the present invention includes, but is not limited to, peptides of 40 and 42 amino acids and are  
15 referred to, respectively, as Abeta40 (or  $A\beta_{40}$ ) (SEQ ID NO: 1) and Abeta42 (or  $A\beta_{42}$ ) (SEQ ID NO: 2).

"C99" refers to a peptide that contains the Abeta region plus the cytoplasmic tail of APP (SEQ ID NO: 3). As used herein, the term also includes the C99 London  
20 sequence, which carries the London FAD Alzheimer's associated mutation (SEQ ID NO: 4) (Goate, A., et al (1991). *Nature* 349: 704-706). Abeta and C99 peptides are well known to one of skill in the art (see, for example, Golde et al., *Science* 255:728-730 (1992); Coughlan, C.M. and Breen, K.C. (2000). *Pharmacol. and Ther.* 86: 111-144).

25 "UAS" region as used herein refers to an upstream activating sequence recognized by the GAL-4 transcriptional activator.

As used herein, a "signal sequence" refers to a short sequence of amino acids that determines the eventual location of a protein in a cell, for example, the N-terminal  
30 sequence or 20 or so amino acids that directs nascent secretory and transmembrane proteins to the endoplasmic reticulum. It is contemplated herein that any conventional

signal sequence familiar to one of skill in the art may be used to ensure transfer of the encoded C99 or Abeta proteins through the secretory pathway, including, but not limited to, the signal sequence of endogenous *Drosophila* Appl or presenilin, or of the windbeutel gene, encoding for a ER (endoplasmic reticulum) resident protein (Konsolaki and  
 5 Schupbach, *Genes & Dev.* 12: 120-131 (1998)), or the human pre-proenkephaline gene signal (SEQ ID NO: 5).

As used herein, a "control" fly refers to a larva or fly that is of the same genotype as larvae or flies used in the methods of the present invention except that the control larva  
 10 or fly does not carry the mutation being tested for modification of phenotype, or is not administered candidate compounds.

As used herein, a "control subject" refers to an organism that does not suffer from a condition associated with abnormalities in the APP pathway.

As used herein, a "Drosophila transformation vector" is a DNA plasmid that contains transposable element sequences and can mediate integration of a piece of DNA  
 15 in the genome of the organism. This technology is familiar to one of skill in the art.

As the term is used herein, the "rough eye" phenotype is characterized by disorganization of ommatidia and inter-ommatidial bristles and can be caused by  
 20 degeneration of neuronal cells. This phenotype is visible through a dissecting stereo-microscope.

As the term is used herein, the "concave wing" phenotype is characterized by abnormal folding of the fly wing such that the wings are bent upwards along their long  
 25 margins.

As used herein, a "locomotory defect" refers to a phenotype wherein flies display  
 30 impaired responses to mechanical agitation compared to wild type flies in conventional locomotory activity assays.

As used herein, the following and related phrases, pathological conditions associated with abnormalities in the APP pathway, conditions associated with abnormal regulation of the APP pathway, conditions related to Alzheimer's Disease, pathological conditions associated with defects in the APP pathway, all include, but are not limited to, Alzheimer's Disease, and include those conditions characterized by degeneration and eventual death of neurons in brain clusters controlling memory, cognition and behavior.

"Therapeutically effective amount" refers to that amount of active ingredient, for example compound or gene product which ameliorates the symptoms of the condition being treated.

Methods of obtaining transgenic organisms, including transgenic *Drosophila*, are well known to one skilled in the art. For example, a commonly used reference for P-element mediated transformation is Spradling, 1986. P element mediated transformation. In *Drosophila: A practical approach* (ed. D. B. Roberts), pp175-197. IRL Press, Oxford, UK)). The EP element technology refers to a binary system, utilizing the yeast Gal4 transcriptional activator, that is used to ectopically regulate the transcription of endogenous *Drosophila* genes. This technology is described in : Brand and Perrimon, 1993. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* 118, pp401-415 and in : Rorth et al, 1998. Systematic gain-of-function genetics in *Drosophila*. *Development*, 125(6), pp1049—1057.

The present invention discloses a transgenic fly, *Drosophila melanogaster*, that contains in its genome a DNA sequence encoding a polypeptide comprising the beta amyloid portion (SEQ ID NO:1 or SEQ ID NO: 2) or C99 portion of the human *APP* gene (SEQ ID NO: 3 or SEQ ID NO: 4) which is fused at its N-terminus according to conventional methods to a signal peptide sequence, for example, SEQ ID NO:5, to ensure transfer of the encoded polypeptide through the secretory pathway. The fused DNA sequences are operably linked to tissue-specific expression control sequences such as promoter regions or upstream activating sequences (UAS), depending on the

expression system utilized. These expression control sequences include those that are specific for neural tissue in the fly and include organs such as the eye, wing, notum, brain, CNS and PNS. Under the control of these tissue specific control sequences , encoded peptides are transcribed to form mRNA which is translated into detectable levels of beta amyloid or C99 peptide and which causes altered phenotypes in the flies. By assaying for changes in these phenotypes, these flies can be used to identify genes or compounds that may affect the APP pathway and may provide insight into the molecular and biochemical mechanisms of the APP pathway and Alzheimer's Disease.

10. Conventional expression control systems may be used to achieve ectopic expression of proteins of interest, including the beta amyloid and C99 peptides of the present invention. Such expression may result in the disturbance of biochemical pathways and the generation of altered phenotypes. One such expression control system involves direct fusion of the DNA sequence to expression control sequences of tissue-specifically expressed genes, such as promoters or enhancers. Another expression control system that may be used is the binary Gal4-transcriptional activation system (Brand and Perrimon, *Development* 118:401-415 (1993)).

The Gal4 system uses the yeast transcriptional activator Gal4, to drive the expression of a gene of interest in a tissue specific manner. The Gal4 gene has been randomly inserted into the fly genome, using a conventional transformation system, so that it has come under the control of genomic enhancers that drive expression in a temporal and tissue-specific manner. Individual strains of flies have been established, called "drivers", that carry those insertions (Brand and Perrimon, *Development* 118:401-415 (1993)).

In the Gal4 system, a gene of interest is cloned into a transformation vector, so that its transcription is under the control of the *UAS* sequence (Upstream Activating Sequences), the Gal4-responsive element. When a fly strain that carries the *UAS*-gene of interest sequence is crossed to a fly strain that expresses the *Gal4* gene under the control of a tissue specific enhancer, the gene will be expressed in a tissue specific pattern.



In order to generate phenotypes that are easily visible in adult tissues and can thus be used in genetic screens, Gal4 “drivers” that drive expression in later stages of the fly development may be used in the present invention. Using these drivers, expression would result in possible defects in the wings, the eyes, the legs, different sensory organs and the brain. These “drivers” include, for example, *apterous*-Gal4 (wings), *elav*-Gal4 (CNS), *sevenless*-Gal4, *eyeless*-Gal4 and *pGMR*-Gal4 (eyes). In addition, since *Appl*, the fly homologue of *APP*, is exclusively expressed in neural tissue, “driver” strains in which at least a subset of expression is directed to a part of the nervous system, are preferred. This includes the brain specific *7B*-Gal4 driver. Descriptions of the Gal4 lines and notes about their specific expression patterns is available in Flybase (<http://flybase.bio.indiana.edu>) .

Various DNA constructs may be used to generate the transgenic *Drosophila melanogaster* of the present invention. For example, the construct may contain the beta amyloid or C99 portion of the human *APP* gene fused to the pre-proenkephaline gene signal peptide sequence and operably linked to the eye-specific promoter, GMR. In another example, the construct may contain the beta amyloid portion or C99 of the human *APP* gene fused to the human pre-proenkephaline gene signal peptide sequence cloned into the pUAST vector (Brand and Perrimon, *Development* 118:401-415 (1993)) which places the UAS sequence upstream of the transcribed region. Insertion of these constructs into the fly genome may occur through P-element recombination, Hobo element recombination (Blackman et al., *EMBO J.* 8:211-217 (1989)), homologous recombination (Rong and Golic, *Science* 288:2013-2018 (2000)) or other standard techniques known to one of skill in the art.

As discussed above, an ectopically expressed gene may result in an altered phenotype by disruption of a particular biochemical pathway. Mutations in genes acting in the same biochemical pathway are expected to cause modification of the altered phenotype. Thus, the flies of the present invention can be used to identify genes acting in the *APP* pathway by crossing a C99 or Abeta transgenic fly with a fly containing a

mutation in a known or predicted gene; and screening progeny of the crosses for flies that display quantitative or qualitative modification of the altered phenotype of the C99 or Abeta transgenic fly, as compared to controls. Thus, this system is extremely beneficial for the elucidation of the function of processed APP gene products, as well as the identification of other genes that directly or indirectly interact with them. Mutations that can be screened include, but are not limited to, loss-of-function alleles of known genes, deletion strains, "enhancer-trap" strains generated by the P-element and gain-of-function mutations generated by random insertions into the Drosophila genome of a Gal4-inducible construct that can activate the ectopic expression of genes in the vicinity of its insertion. It is contemplated herein that genes involved in the APP pathway can be identified in this manner and these genes can then serve as targets for the development of therapeutics to treat conditions associated with abnormalities in the APP pathway, leading to diseases, including but not limited to, Alzheimer's Disease.

The C99 and Abeta transgenic flies of the present invention may also be used in a method to identify compounds that may modify the APP pathway and may thus prove useful for the treatment of conditions discussed above. Said method may comprise administering candidate compounds to C99 or Abeta transgenic flies and then assaying for changes in the phenotype of the C99 or Abeta transgenic fly as compared to the phenotype of control C99 or Abeta transgenic flies that have not been administered the compound. For example, using conventional methods, candidate compounds can be fed to larvae expressing a beta amyloid or C99. The larvae can then be grown to the adult stage and modification of the C99 or Abeta-induced phenotype assayed. Candidate compounds may also be fed to adult flies and modifications of phenotype assayed.

The mechanism of action of compounds thus identified may be examined by comparing the phenotypes produced by genetic manipulation with those induced by the administration of a compound of interest. Such compounds include those that may ameliorate or worsen the altered phenotype created in the transgenic flies. Expression of

a compound-induced phenotype similar to one associated with a known genetic modification would suggest that the compound has an effect on the same pathway that the genetic modification is affecting.

5           In addition to screening compounds in the transgenic flies of the present invention, such compounds may also be further assayed by employing in vitro and other in vivo models of AD using conventional methods. For example, numerous cell lines may be used as in vitro models of AD and are familiar to one of skill in the art, including, for example, the cell lines described in Xia et al, 1997 PNAS USA 94 (15):8208-13. In  
10       vivo models also exist and include, for example, the mouse model of AD disclosed in WO 94/00569.

          Elucidation of the mechanism of action of compounds which affect the action of beta amyloid or C99 in the transgenic flies disclosed herein may also be performed using  
15       RNA profiling on chips (Affymetrix, Santa Clara) or using other conventional methods. For example, the RNA profiles of flies which have been administered candidate compounds may be assayed and compared to those of flies which have been genetically modified. Similar profiles would suggest that the compound acts in some way on the beta amyloid or C99 affected pathway.

          It is contemplated herein that, in yet another aspect, the invention pertains to a method for identifying genes involved in the onset or progression of Alzheimer's Disease and whose protein products might serve as potential markers for AD, said method comprising identifying genes that are involved in the pathways regulated by the  
25       transcription factors encoded by the human sequences hCP50765 (SEQ ID NO. 35, encoded by the EGR2 gene), and hCP41313 (Seq ID NO 15, SEQ ID NO17 or SEQ ID NO 53, encoded by the human homolog of the Drosophila nocA gene), which human sequences are homologs of Drosophila genetic modifiers identified as described herein and are located close to the area of human chromosome 10 that is shown to have genetic  
30       linkage to Alzheimer's Disease. Identification of such genes, regulated by the above mentioned transcription factors, may be achieved using conventional methods, including

but not limited to, a technology called SELEX, referenced in Tuerk and Gold, 1990, Science 249, 505-510 and Brown and Gold, 1995, Biochemistry 34, 14765-14774. For example, genes that are regulated by a specific transcription factor can be identified by determining the target DNA sequence of the specific transcription factor. Such target  
5 sequence identification can be achieved by different methods, including but not limited to SELEX. Once the target sequence is identified, the presence of this sequence in the upstream regulatory regions of known and predicted genes can be determined, using bio-informatics tools well known to one of skill in the art. Genes containing the target sequence in their upstream regulatory regions can be expected to be regulated by the  
10 specific transcription factor.

It is contemplated that compounds which can affect (e.g. inhibit or promote) the function or expression of proteins encoded by the human homologs of genetic modifiers identified according to the present invention may be useful to treat Alzheimer's Disease  
15 or other conditions associated with defects in the regulation of the APP pathway. In addition, it is also contemplated that, using conventional methods, antisense oligonucleotides, ribozymes, triple helix DNA and/or double stranded RNA of therapeutic value may be created based on the nucleotide sequences of these human homologs of genetic modifiers. The therapeutic use of antibodies directed to the  
20 polypeptides encoded by human homologs of genetic modifiers and created using conventional methods is also contemplated herein. Thus, an additional aspect of the invention relates to the administration of a pharmaceutical composition, in conjunction with a pharmaceutically acceptable carrier, excipient or diluent, for the treatment of Alzheimer's Disease and related conditions. Such pharmaceutical compositions may  
25 comprise the compounds, antisense oligonucleotides, ribozymes, triple helix DNA, double stranded RNA and/or antibodies discussed above. The compositions may also contain expression products of human homologs of the genetic modifiers (e.g. polypeptides or fragments thereof) identified according to the present invention. The compositions may be administered alone or in combination with at least one other agent,  
30 such as stabilizing compound, which may be administered in any sterile, biocompatible pharmaceutical carrier, including, but not limited to, saline, buffered saline, dextrose, and

water. The compositions may be administered to a subject in need thereof alone, or in combination with other agents, drugs or hormones.

The pharmaceutical compositions encompassed by the invention may be administered by any number of routes including, but not limited to, oral, intravenous, intramuscular, intra-articular, intra-arterial, intramedullary, intrathecal, intraventricular, transdermal, subcutaneous, intraperitoneal, intranasal, enteral, topical, sublingual, or rectal means.

In addition to the active ingredients, these pharmaceutical compositions may contain suitable pharmaceutically-acceptable carriers comprising excipients and auxiliaries which facilitate processing of the active compounds into preparations which can be used pharmaceutically. Further details on techniques for formulation and administration may be found in the latest edition of Remington's Pharmaceutical Sciences (Maack Publishing Co., Easton, Pa.).

Pharmaceutical compositions for oral administration can be formulated using pharmaceutically acceptable carriers well known in the art in dosages suitable for oral administration. Such carriers enable the pharmaceutical compositions to be formulated as tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions, and the like, for ingestion by the patient.

Pharmaceutical preparations for oral use can be obtained through combination of active compounds with solid excipient, optionally grinding a resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries, if desired, to obtain tablets or dragee cores. Suitable excipients are carbohydrate or protein fillers, such as sugars, including lactose, sucrose, mannitol, or sorbitol; starch from corn, wheat, rice, potato, or other plants; cellulose, such as methyl cellulose, hydroxypropylmethylcellulose, or sodium carboxymethylcellulose; gums including arabic and tragacanth; and proteins such as gelatin and collagen. If desired, disintegrating or solubilizing agents may

be added, such as the cross-linked polyvinyl pyrrolidone, agar, alginic acid, or a salt thereof, such as sodium alginate.

Dragee cores may be used in conjunction with suitable coatings, such as concentrated sugar solutions, which may also contain gum arabic, talc, polyvinylpyrrolidone, carbopol gel, polyethylene glycol, and/or titanium dioxide, lacquer solutions, and suitable organic solvents or solvent mixtures. Dyestuffs or pigments may be added to the tablets or dragee coatings for product identification or to characterize the quantity of active compound, i.e., dosage.

Pharmaceutical preparations which can be used orally include push-fit capsules made of gelatin, as well as soft, sealed capsules made of gelatin and a coating, such as glycerol or sorbitol. Push-fit capsules can contain active ingredients mixed with a filler or binders, such as lactose or starches, lubricants, such as talc or magnesium stearate, and, optionally, stabilizers. In soft capsules, the active compounds may be dissolved or suspended in suitable liquids, such as fatty oils, liquid, or liquid polyethylene glycol with or without stabilizers.

Pharmaceutical formulations suitable for parenteral administration may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hanks' solution, Ringer's solution, or physiologically buffered saline. Aqueous injection suspensions may contain substances which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol, or dextran. Additionally, suspensions of the active compounds may be prepared as appropriate oily injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acid esters, such as ethyl oleate or triglycerides, or liposomes. Non-lipid polycationic amino polymers may also be used for delivery. Optionally, the suspension may also contain suitable stabilizers or agents which increase the solubility of the compounds to allow for the preparation of highly concentrated solutions.

For topical or nasal administration, penetrants appropriate to the particular barrier to be permeated are used in the formulation. Such penetrants are generally known in the art.

5           The pharmaceutical compositions of the present invention may be manufactured in a manner that is known in the art, e.g., by means of conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping, or lyophilizing processes.

10           The pharmaceutical composition may be provided as a salt and can be formed with many acids, including but not limited to, hydrochloric, sulfuric, acetic, lactic, tartaric, malic, succinic, etc. Salts tend to be more soluble in aqueous or other protonic solvents than are the corresponding free base forms. In other cases, the preferred preparation may be a lyophilized powder which may contain any or all of the following:  
15   1-50 mM histidine, 0.1%-2% sucrose, and 2-7% mannitol, at a pH range of 4.5 to 5.5, that is combined with buffer prior to use.

After pharmaceutical compositions have been prepared, they can be placed in an appropriate container and labeled for treatment of an indicated condition. For  
20   administration of the compounds or gene products identified according to the present invention, such labeling would include amount, frequency, and method of administration.

Pharmaceutical compositions suitable for use in the invention include compositions wherein the active ingredients are contained in an effective amount to  
25   achieve the intended purpose. The determination of an effective dose is well within the capability of those skilled in the art.

For any compound, the therapeutically effective dose can be estimated initially either in cell culture assays, e.g., of neoplastic cells, or in animal models, usually mice,  
30   rabbits, dogs, or pigs. The animal model may also be used to determine the appropriate

concentration range and route of administration. Such information can then be used to determine useful doses and routes for administration in humans.

5 A "therapeutically effective dose" refers to that amount of active ingredient, for example compound or gene product which ameliorates the symptoms or condition. Therapeutic efficacy and toxicity may be determined by standard pharmaceutical procedures in cell cultures or experimental animals, e.g., ED50 (the dose therapeutically effective in 50% of the population) and LD50 (the dose lethal to 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index, and it can be expressed as the ratio, LD50/ED50. Pharmaceutical compositions which exhibit large therapeutic indices are preferred. The data obtained from cell culture assays and animal studies is used in formulating a range of dosage for human use. The dosage contained in such compositions is preferably within a range of circulating concentrations that include the ED50 with little or no toxicity. The dosage varies within this range depending upon the dosage form employed, sensitivity of the patient, and the route of administration.

15 The exact dosage will be determined by the practitioner, in light of factors related to the subject that requires treatment. Dosage and administration are adjusted to provide sufficient levels of the active moiety or to maintain the desired effect. Factors which may be taken into account include the severity of the disease state, general health of the subject, age, weight, and gender of the subject, diet, time and frequency of administration, drug combination(s), reaction sensitivities, and tolerance/response to therapy. Long-acting pharmaceutical compositions may be administered every 3 to 4 days, every week, or once every two weeks depending on half-life and clearance rate of the particular formulation.

25 Normal dosage amounts may vary from 0.1 to 100,000 micrograms, up to a total dose of about 1 g, depending upon the route of administration. Guidance as to particular dosages and methods of delivery is provided in the literature and generally available to practitioners in the art. Those skilled in the art will employ different formulations for nucleotides than for proteins or their inhibitors. Similarly, delivery of polynucleotides or



polypeptides will be specific to particular cells, conditions, locations, etc.

Pharmaceutical formulations suitable for oral administration of proteins are described, e.g., in U.S. Patents 5,008,114; 5,505,962; 5,641,515; 5,681,811; 5,700,486; 5,766,633; 5,792,451; 5,853,748; 5,972,387; 5,976,569; and 6,051,561.

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It is also contemplated herein that a method for the diagnosis of pathological conditions associated with abnormalities in the APP pathway in a subject, including but not limited to Alzheimer's Disease, is possible given the data of Table 1. For example, the method may comprise measuring the level of polypeptides encoded by any one or more of the human genetic homologs of the genes of Table 1 in a biological sample from a subject, wherein an abnormal level of any one or more of said polypeptides relative to the level thereof in a normal subject is diagnostic of said conditions. Such an assay could be performed using conventional technologies familiar to one of skill in the art.

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In another embodiment, nucleic acids comprising a sequence encoding a human homolog of a genetic modifier or functional derivative thereof are administered to promote APP pathway function, by way of gene therapy. Gene therapy refers to therapy performed by the administration of a nucleic acid to a subject. In this embodiment of the invention, the nucleic acid produces its encoded protein that mediates a therapeutic effect by promoting normal APP pathway function.

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Any of the methods for gene therapy available in the art can be used according to the present invention. Exemplary methods are described below.

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In a preferred aspect, the therapeutic comprises the nucleic acid for a genetic modifier that is part of an expression vector that expresses a genetic modifier protein or fragment or chimeric protein thereof in a suitable host. In particular, such a nucleic acid has a promoter operably linked to the specific genetic modifier protein coding region, said promoter being inducible or constitutive, and, optionally, tissue-specific. In another particular embodiment, a nucleic acid molecule is used in which the modifier protein coding sequences and any other desired sequences are flanked by regions that promote

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homologous recombination at a desired site in the genome, thus providing for intrachromosomal expression of the modifier nucleic acid (Koller and Smithies, 1989, Proc. Natl. Acad. Sci. USA 86:8932-8935; Zijlstra et al., 1989, Nature 342:435-438).

5           Delivery of the nucleic acid into a patient may be either direct, in which case the patient is directly exposed to the nucleic acid or nucleic acid-carrying vector, or indirect, in which case, cells are first transformed with the nucleic acid in vitro, then transplanted into the patient. These two approaches are known, respectively, as *in vivo* or *ex vivo* gene therapy.

10           In a specific embodiment, the nucleic acid is directly administered *in vivo*, where it is expressed to produce the encoded product. This can be accomplished by any of numerous methods known in the art, e.g., by constructing it as part of an appropriate nucleic acid expression vector and administering it so that it becomes intracellular, e.g.,  
15           by infection using a defective or attenuated retroviral or other viral vector (see, e.g., U.S. Pat. No. 4,980,286 and others mentioned *infra*), or by direct injection of naked DNA, or by use of microparticle bombardment (e.g., a gene gun; Biolistic, Dupont), or coating with lipids or cell-surface receptors or transfecting agents, encapsulation in liposomes, microparticles, or microcapsules, or by administering it in linkage to a peptide which is  
20           known to enter the nucleus, by administering it in linkage to a ligand subject to receptor-mediated endocytosis (see e.g., U.S. Patents 5,166,320; 5,728,399; 5,874,297; and 6,030,954, all of which are incorporated by reference herein in their entirety) (which can be used to target cell types specifically expressing the receptors), etc. In another embodiment, a nucleic acid-ligand complex can be formed in which the ligand comprises  
25           a fusogenic viral peptide to disrupt endosomes, allowing the nucleic acid to avoid lysosomal degradation. In yet another embodiment, the nucleic acid can be targeted in vivo for cell specific uptake and expression, by targeting a specific receptor (see, e.g., PCT Publications WO 92/06180; WO 92/22635; WO92/20316; WO93/14188; and WO 93/20221). Alternatively, the nucleic acid can be introduced intracellularly and

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incorporated within host cell DNA for expression, by homologous recombination (see, e.g., U.S. Patents 5,413,923; 5,416,260; and 5,574,205; and Zijlstra et al., 1989, Nature 342:435-438).

5 In a specific embodiment, a viral vector that contains a modifier nucleic acid is used. For example, a retroviral vector can be used (see, e.g., U.S. Patents 5,219,740; 5,604,090; and 5,834,182). These retroviral vectors have been modified to delete retroviral sequences that are not necessary for packaging of the viral genome and integration into host cell DNA. The modifier nucleic acid to be used in gene therapy is  
10 cloned into the vector, which facilitates delivery of the gene into a patient.

Adenoviruses are other viral vectors that can be used in gene therapy.

Adenoviruses are especially attractive vehicles for delivering genes to respiratory epithelia. Adenoviruses naturally infect respiratory epithelia where they cause a mild  
15 disease. Other targets for adenovirus-based delivery systems are liver, the central nervous system, endothelial cells, and muscle. Adenoviruses have the advantage of being capable of infecting non-dividing cells. Methods for conducting adenovirus-based gene therapy are described in, e.g., U.S. Patents 5,824,544; 5,868,040; 5,871,722; 5,880,102; 5,882,877; 5,885,808; 5,932,210; 5,981,225; 5,994,106; 5,994,132; 5,994,134; 6,001,557;  
20 and 6,033,8843, all of which are incorporated by reference herein in their entirety.

Adeno-associated virus (AAV) has also been proposed for use in gene therapy. Methods for producing and utilizing AAV are described, e.g., in U.S. Patents 5,173,414; 5,252,479; 5,552,311; 5,658,785; 5,763,416; 5,773,289; 5,843,742; 5,869,040; 5,942,496;  
25 and 5,948,675, all of which are incorporated by reference herein in their entirety.

Another approach to gene therapy involves transferring a gene to cells in tissue culture by such methods as electroporation, lipofection, calcium phosphate mediated transfection, or viral infection. Usually, the method of transfer includes the transfer of a  
30 selectable marker to the cells. The cells are then placed under selection to isolate those

cells that have taken up and are expressing the transferred gene. Those cells are then delivered to a patient.

In this embodiment, the nucleic acid is introduced into a cell prior to administration in vivo of the resulting recombinant cell. Such introduction can be carried out by any method known in the art, including but not limited to transfection, electroporation, microinjection, infection with a viral or bacteriophage vector containing the nucleic acid sequences, cell fusion, chromosome-mediated gene transfer, microcell-mediated gene transfer, spheroplast fusion, etc. Numerous techniques are known in the art for the introduction of foreign genes into cells and may be used in accordance with the present invention, provided that the necessary developmental and physiological functions of the recipient cells are not disrupted. The technique should provide for the stable transfer of the nucleic acid to the cell, so that the nucleic acid is expressible by the cell and preferably heritable and expressible by its cell progeny.

The resulting recombinant cells can be delivered to a patient by various methods known in the art. In a preferred embodiment, epithelial cells are injected, e.g., subcutaneously. In another embodiment, recombinant skin cells may be applied as a skin graft onto the patient. Recombinant blood cells (e.g., hematopoietic stem or progenitor cells) are preferably administered intravenously. The amount of cells envisioned for use depends on the desired effect, patient state, etc., and can be determined by one skilled in the art.

Cells into which a nucleic acid can be introduced for purposes of gene therapy encompass any desired, available cell type, and include but are not limited to epithelial cells, endothelial cells, keratinocytes, fibroblasts, muscle cells, hepatocytes, blood cells such as T lymphocytes, B lymphocytes, monocytes, macrophages, neutrophils, eosinophils, megakaryocytes, granulocytes; various stem or progenitor cells, in particular hematopoietic stem or progenitor cells, e.g., as obtained from bone marrow, umbilical cord blood, peripheral blood, fetal liver, etc.

In a preferred embodiment, the cell used for gene therapy is autologous to the patient.

In an embodiment in which recombinant cells are used in gene therapy, a modifier nucleic acid is introduced into the cells such that it is expressible by the cells or their progeny, and the recombinant cells are then administered in vivo for therapeutic effect. In a specific embodiment, stem or progenitor cells are used. Any stem-and/or progenitor cells that can be isolated and maintained in vitro can potentially be used in accordance with this embodiment of the present invention. Such stem cells include but are not limited to hematopoietic stem cells (HSC), stem cells of epithelial tissues such as the skin and the lining of the gut, embryonic heart muscle cells, liver stem cells (see, e.g., WO 94/08598), and neural stem cells (Stemple and Anderson, 1992, Cell 71:973-985).

Epithelial stem cells (ESCs) or keratinocytes can be obtained from tissues such as the skin and the lining of the gut by known procedures (Rheinwald, 1980, Meth. Cell Bio. 21A:229). In stratified epithelial tissue such as the skin, renewal occurs by mitosis of stem cells within the germinal layer, the layer closest to the basal lamina. Stem cells within the lining of the gut provide for a rapid renewal rate of this tissue. ESCs or keratinocytes obtained from the skin or lining of the gut of a patient or donor can be grown in tissue culture (Pittelkow and Scott, 1986, Mayo Clinic Proc. 61:771). If the ESCs are provided by a donor, a method for suppression of host versus graft reactivity (e.g., irradiation, drug or antibody administration to promote moderate immunosuppression) can also be used.

With respect to hematopoietic stem cells (HSC), any technique that provides for the isolation, propagation, and maintenance in vitro of HSC can be used in this embodiment of the invention. Techniques by which this may be accomplished include (a) the isolation and establishment of HSC cultures from bone marrow cells isolated from the future host, or a donor, or (b) the use of previously established long-term HSC cultures, which may be allogeneic or xenogeneic. Non-autologous HSC are used preferably in conjunction with a method of suppressing transplantation immune reactions of the future

host/patient. In a particular embodiment of the present invention, human bone marrow cells can be obtained from the posterior iliac crest by needle aspiration (see, e.g., Kodo et al., 1984, J. Clin. Invest. 73:1377-1384). In a preferred embodiment of the present invention, the HSCs can be made highly enriched or in substantially pure form. This enrichment can be accomplished before, during, or after long-term culturing, and can be done by any techniques known in the art. Long-term cultures of bone marrow cells can be established and maintained by using, for example, modified Dexter cell culture techniques (Dexter et al., 1977, J. Cell Physiol. 91:335) or Witlock-Witte culture techniques (Witlock and Witte, 1982, Proc. Natl. Acad. Sci. USA 79:3608-3612).

In a specific embodiment, the nucleic acid to be introduced for purposes of gene therapy comprises an inducible promoter operably linked to the coding region, such that expression of the nucleic acid is controllable by controlling the presence or absence of the appropriate inducer of transcription.

A further embodiment of the present invention relates to the therapeutic use of a purified antibody or a fragment thereof for the treatment of conditions associated with abnormalities in the APP pathway, including but not limited to, AD. It is contemplated that the purified antibody or a fragment thereof specifically binds to a polypeptide that comprises the amino acid sequence of any of the human homologs of the genetic modifiers identified in Table 1, preferably, the polypeptides of human homologs located on chromosome 10 disclosed herein, most preferably, the polypeptide encoded by SEQ ID NO: 15, SEQ ID NO: 17 or SEQ ID NO 53, i.e. the curated noc A sequences, or to a fragment of said polypeptides. A preferred embodiment relates to a fragment of such an antibody, which fragment is an Fab or F(ab')<sub>2</sub> fragment. In particular, the antibody can be a polyclonal antibody or a monoclonal antibody.

Described herein are methods for the production of antibodies capable of specifically recognizing one or more differentially expressed gene epitopes. Such antibodies may include, but are not limited to polyclonal antibodies, monoclonal antibodies (mAbs), humanized or chimeric antibodies, single chain antibodies, Fab

fragments, F(ab')<sub>2</sub> fragments, fragments produced by a Fab expression library, anti-idiotypic (anti-Id) antibodies, and epitope-binding fragments of any of the above. Such antibodies may be used, for example, in the detection of a fingerprint, target, gene in a biological sample, or, alternatively, as a method for the inhibition of abnormal target gene activity. Thus, such antibodies may be utilized as part of Alzheimer's disease treatment methods, and/or may be used as part of diagnostic techniques whereby patients may be tested for abnormal levels of a modifier polypeptide, or for the presence of abnormal forms of a modifier polypeptide.

For the production of antibodies to a specific modifier polypeptide, various host animals may be immunized by injection with the polypeptide, or a portion thereof.. Such host animals may include but are not limited to rabbits, mice, and rats, to name but a few. Various adjuvants may be used to increase the immunological response, depending on the host species, including but not limited to Freund's (complete and incomplete), mineral gels such as aluminum hydroxide, surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanin, dinitrophenol, and potentially useful human adjuvants such as BCG (bacille Calmette-Guerin) and *Corynebacterium parvum*.

Polyclonal antibodies are heterogeneous populations of antibody molecules derived from the sera of animals immunized with an antigen, such as target gene product, or an antigenic functional derivative thereof. For the production of polyclonal antibodies, host animals such as those described above, may be immunized by injection with a modifier polypeptide, or a portion thereof, supplemented with adjuvants as also described above.

Monoclonal antibodies, which are homogeneous populations of antibodies to a particular antigen, may be obtained by any technique that provides for the production of antibody molecules by continuous cell lines in culture. These include, but are not limited to the hybridoma technique of Kohler and Milstein, (1975, *Nature* 256:495-497; and U.S. Pat. No. 4,376,110), the human B-cell hybridoma technique (Kosbor et al., 1983,

Immunology Today 4:72; Cole et al., 1983, Proc. Natl. Acad. Sci. USA 80:2026-2030), and the EBV-hybridoma technique (Cole et al., 1985, Monoclonal Antibodies And Cancer Therapy, Alan R. Liss, Inc., pp. 77-96). Such antibodies may be of any immunoglobulin class including IgG, IgM, IgE, IgA, IgD and any subclass thereof. The hybridoma producing the mAb of this invention may be cultivated in vitro or in vivo. Production of high titers of mAbs in vivo makes this the presently preferred method of production.

In addition, techniques developed for the production of "chimeric antibodies" (Morrison et al., 1984, Proc. Natl. Acad. Sci., 81:6851-6855; Neuberger et al., 1984, Nature, 312:604-608; Takeda et al., 1985, Nature, 314:452-454) by splicing the genes from a mouse antibody molecule of appropriate antigen specificity together with genes from a human antibody molecule of appropriate biological activity can be used. A chimeric antibody is a molecule in which different portions are derived from different animal species, such as those having a variable or hypervariable region derived from a murine mAb and a human immunoglobulin constant region.

Alternatively, techniques described for the production of single chain antibodies (U.S. Pat. No. 4,946,778; Bird, 1988, Science 242:423-426; Huston et al., 1988, Proc. Natl. Acad. Sci. USA 85:5879-5883; and Ward et al., 1989, Nature 334:544-546) can be adapted to produce differentially expressed gene-single chain antibodies. Single chain antibodies are formed by linking the heavy and light chain fragments of the Fv region via an amino acid bridge, resulting in a single chain polypeptide.

Most preferably, techniques useful for the production of "humanized antibodies" can be adapted to produce antibodies to the polypeptides, fragments, derivatives, and functional equivalents disclosed herein. Such techniques are disclosed in U.S. Patent Nos. 5,932, 448; 5,693,762; 5,693,761; 5,585,089; 5,530,101; 5,910,771; 5,569,825; 5,625,126; 5,633,425; 5,789,650; 5,545,580; 5,661,016; and 5,770,429, the disclosures of all of which are incorporated by reference herein in their entirety.



Antibody fragments that recognize specific epitopes may be generated by known techniques. For example, such fragments include but are not limited to: the  $F(ab')_2$  fragments which can be produced by pepsin digestion of the antibody molecule and the Fab fragments which can be generated by reducing the disulfide bridges of the  $F(ab')_2$  fragments. Alternatively, Fab expression libraries may be constructed (Huse et al., 1989, Science, 246:1275-1281) to allow rapid and easy identification of monoclonal Fab fragments with the desired specificity.

An antibody of the present invention can be preferably used in a method for the diagnosis of a condition associated with abnormal APP pathway regulation and/or Alzheimer's Disease in a subject, or to identify a subject with a predisposition to said conditions, which comprises: measuring the amount of a polypeptide comprising the amino acid sequence of any of the human homologs of the genetic modifiers identified in Table 1, preferably, the polypeptides of human homologs located on chromosome 10 disclosed herein, most preferably, the polypeptide encoded by SEQ ID NO: 15, SEQ ID NO: 17 or SEQ ID NO 53, i.e. the curated noc A sequences, or fragments thereof, in an appropriate tissue or cell from a subject wherein the presence of an elevated amount of said polypeptide or fragments thereof, relative to the amount of said polypeptide or fragments thereof in the respective tissue from a control subject is diagnostic of said condition. Such a method forms a further embodiment of the present invention. Preferably, said detecting step comprises contacting said appropriate tissue or cell with an antibody which specifically binds to a polypeptide that comprises the amino acid sequence of any one or more of the polypeptides discussed above or a fragment thereof and detecting specific binding of said antibody with a polypeptide in said appropriate tissue or cell, wherein detection of specific binding to a polypeptide indicates the presence of any one or more of said polypeptides or a fragment thereof.

Particularly preferred, for ease of detection, is the sandwich assay, of which a number of variations exist, all of which are intended to be encompassed by the present invention.

For example, in a typical forward assay, unlabeled antibody is immobilized on a solid substrate and the sample to be tested brought into contact with the bound molecule. After a suitable period of incubation, for a period of time sufficient to allow formation of an antibody-antigen binary complex. At this point, a second antibody, labeled with a reporter molecule capable of inducing a detectable signal, is then added and incubated, allowing time sufficient for the formation of a ternary complex of antibody-antigen-labeled antibody. Any unreacted material is washed away, and the presence of the antigen is determined by observation of a signal, or may be quantitated by comparing with a control sample containing known amounts of antigen. Variations on the forward assay include the simultaneous assay, in which both sample and antibody are added simultaneously to the bound antibody, or a reverse assay in which the labeled antibody and sample to be tested are first combined, incubated and added to the unlabeled surface bound antibody. These techniques are well known to those skilled in the art, and the possibility of minor variations will be readily apparent. As used herein, "sandwich assay" is intended to encompass all variations on the basic two-site technique. For the immunoassays of the present invention, the only limiting factor is that the labeled antibody be an antibody that is specific for modifier polypeptide or a fragment thereof.

The most commonly used reporter molecules in this type of assay are either enzymes, fluorophore- or radionuclide-containing molecules. In the case of an enzyme immunoassay an enzyme is conjugated to the second antibody, usually by means of glutaraldehyde or periodate. As will be readily recognized, however, a wide variety of different ligation techniques exist, which are well-known to the skilled artisan. Commonly used enzymes include horseradish peroxidase, glucose oxidase, beta-galactosidase and alkaline phosphatase, among others. The substrates to be used with the specific enzymes are generally chosen for the production, upon hydrolysis by the corresponding enzyme, of a detectable color change. For example, p-nitrophenyl phosphate is suitable for use with alkaline phosphatase conjugates; for peroxidase conjugates, 1,2-phenylenediamine or toluidine are commonly used. It is also possible to employ fluorogenic substrates, which yield a fluorescent product rather than the chromogenic substrates noted above. A solution containing the appropriate substrate is

then added to the tertiary complex. The substrate reacts with the enzyme linked to the second antibody, giving a qualitative visual signal, which may be further quantitated, usually spectrophotometrically, to give an evaluation of the amount of modifier protein which is present in the serum sample.

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Alternately, fluorescent compounds, such as fluorescein and rhodamine, may be chemically coupled to antibodies without altering their binding capacity. When activated by illumination with light of a particular wavelength, the fluorochrome-labeled antibody absorbs the light energy, inducing a state of excitability in the molecule, followed by  
 10 emission of the light at a characteristic longer wavelength. The emission appears as a characteristic color visually detectable with a light microscope. Immunofluorescence and EIA techniques are both very well established in the art and are particularly preferred for the present method. However, other reporter molecules, such as radioisotopes, chemiluminescent or bioluminescent molecules may also be employed. It will be readily  
 15 apparent to the skilled artisan how to vary the procedure to suit the required use.

Polynucleotides encoding human homologs of genetic modifiers identified according to the methods of the present invention may be used in a method to diagnose conditions associated with defects in the regulation of the APP pathway, including but  
 20 not limited to Alzheimer's Disease or to identify individuals with a genetic predisposition to such conditions. For example, said method comprises detecting the level of transcription of mRNA transcribed from the gene encoding a human homolog of a genetic modifier disclosed herein in an appropriate tissue or cell from a human, wherein abnormal transcription compared to control levels is diagnostic of said condition or a  
 25 predisposition to said condition. In particular, said genetic modifier comprises the nucleotide sequence of any of the human homologs of the genetic modifiers identified in Table 1, preferably, the polypeptides of human homologs located on chromosome 10 disclosed herein, most preferably, the polypeptides encoded by SEQ ID NO: 15, SEQ ID NO: 17 or SEQ ID NO 53, i.e. the curated noc A sequences, or the polypeptide encoded  
 30 by SEQ ID NO: 35, i.e. the EGR2 sequence, or the polypeptides encoded by SEQ ID NO: 41, or SEQ ID NO: 43, the ankyrin-related sequences.

Detection of a mutated form of a gene encoding a genetic modifier identified according to the methods of the present invention which is associated with a dysfunction will provide a diagnostic tool that can add to, or define, a diagnosis of a disease, or susceptibility to a disease, which results from under-expression, over-expression or altered spatial or temporal expression of the gene. Said diseases may include, but are not limited to, Alzheimer's Disease or other conditions characterized by errors in the regulation of the APP pathway. Individuals carrying mutations in said genes may be detected at the DNA level by a variety of techniques.

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Nucleic acids, in particular mRNA, for diagnosis may be obtained from a subject's cells, such as from blood, urine, saliva, tissue biopsy or autopsy material. The genomic DNA may be used directly for detection or may be amplified enzymatically by using PCR or other amplification techniques prior to analysis. RNA or cDNA may also be used in similar fashion. Deletions and insertions can be detected by a change in size of the amplified product in comparison to the normal genotype. Hybridizing amplified DNA to labeled nucleotide sequences encoding the human homolog of a genetic modifier polypeptide of the present invention can identify point mutations. Perfectly matched sequences can be distinguished from mismatched duplexes by RNase digestion or by differences in melting temperatures. DNA sequence differences may also be detected by alterations in electrophoretic mobility of DNA fragments in gels, with or without denaturing agents, or by direct DNA sequencing (e.g., Myers et al., Science (1985) 230:1242). Sequence changes at specific locations may also be revealed by nuclease protection assays, such as RNase and S1 protection or the chemical cleavage method (see Cotton et al., Proc Natl Acad Sci USA (1985) 85: 4397-4401). In another embodiment, an array of oligonucleotides probes comprising nucleotide sequence encoding a genetic modifier polypeptide of the present invention or fragments of such a nucleotide sequence can be constructed to conduct efficient screening of e.g., genetic mutations. Array technology methods are well known and have general applicability and can be used to address a variety of questions in molecular genetics including gene expression, genetic

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linkage, and genetic variability (see for example: M. Chee et al., Science, Vol 274, pp 610-613 (1996)).

5 The diagnostic assays offer a process for diagnosing or determining a susceptibility to disease through detection of mutation in a human homolog of a modifier gene by the methods described. In addition, such diseases may be diagnosed by methods comprising determining from a sample derived from a subject an abnormally decreased or increased level of polypeptide or mRNA. Decreased or increased expression can be measured at the RNA level using any of the methods well known in the art for the  
10 quantitation of polynucleotides, such as, for example, nucleic acid amplification, for instance PCR, RT-PCR, RNase protection, Northern blotting and other hybridization methods. Assay techniques that can be used to determine levels of a protein, such as a polypeptide of the present invention, in a sample derived from a host are well known to those of skill in the art. Such assay methods include radioimmunoassays, competitive-  
15 binding assays, Western Blot analysis and ELISA assays.

Such hybridization conditions may be highly stringent or less highly stringent, as described above. In instances wherein the nucleic acid molecules are deoxyoligonucleotides ("oligos"), highly stringent conditions may refer, e.g., to washing  
20 in 6X SSC/0.05% sodium pyrophosphate at 37 °C (for 14-base oligos), 48 °C (for 17-base oligos), 55 °C (for 20-base oligos), and 60 °C (for 23-base oligos). Suitable ranges of such stringency conditions for nucleic acids of varying compositions are described in Krause and Aaronson (1991), Methods in Enzymology, 200:546-556 in addition to Maniatis et al., cited above.

25

Thus in another aspect, the present invention relates to a diagnostic kit which comprises:

- (a) a polynucleotide of a human homolog of a genetic modifier identified according to the methods of the present invention, preferably, a polypeptide of a human  
30 homolog located on chromosome 10 disclosed herein, or a fragment thereof,
- (b) a nucleotide sequence complementary to that of (a);

(c) a polypeptide of a genetic modifier of the present invention, preferably the polypeptide of a human homolog of the genetic modifiers identified in Table 1, preferably, the polypeptide of a human homolog located on chromosome 10 disclosed herein, or a fragment thereof; or

5 (d) an antibody to a genetic modifier polypeptide of the present invention, preferably to the polypeptide of a human homolog of the genetic modifiers identified in Table 1, preferably, the polypeptide of a human homolog located on chromosome 10 disclosed herein.

10 It will be appreciated that in any such kit, (a), (b), (c) or (d) may comprise a substantial component. It is also contemplated that such a kit may comprise components directed to one or more of said human homologs. Such a kit will be of use in diagnosing a disease or susceptibility to a disease, particularly to a disease or condition associated with errors in the regulation of the APP pathway including, but not limited to, Alzheimer's Disease.

15 The nucleotide sequences of the human homologs of genetic modifiers of the present invention can also be used for genetic linkage analysis. Since the complete human genome sequence is known, the nucleotide sequence of interest can be specifically mapped to a particular location on an individual human chromosome. The mapping of  
20 relevant sequences to chromosomes according to the present invention is an important first step in correlating those sequences with gene associated disease. Once a sequence has been mapped to a precise chromosomal location, the physical position of the sequence on the chromosome can be correlated with genetic map data. Such data are found in, for example, V. McKusick, Mendelian Inheritance in Man (available on-line  
25 through Johns Hopkins University Welch Medical Library). The relationship between genes and diseases that have been mapped to the same chromosomal region are then identified through linkage analysis (coinheritance of physically adjacent genes). Recent data indicate that there is a region on human chromosome 10 linked to Alzheimer's Disease (Bertram *et al.*, Ertekin-Taner *et al.*, Myers *et al.*, *Science* 290, 2302-2305,  
30 2000), thus, human homologs of genetic modifiers identified according to the methods of

the present invention may be subjected to chromosomal mapping analysis using conventional techniques.

The invention includes an isolated nucleic acid molecule, preferably a DNA molecule, wherein the nucleic acid molecule is the curated sequences of the human nocA homolog set forth in SEQ ID NO: 15, SEQ ID NO:17 or SEQ ID NO 53. Likewise preferred is an isolated nucleic acid molecule, preferably a DNA molecule, encoding a polypeptide comprising the amino acid sequence encoded by the sequence of EGR2, set forth in SEQ ID NO:35. Likewise preferred is an isolated nucleic acid molecule, preferably a DNA molecule, encoding a polypeptide consisting of the amino acid sequences encoded by any of the sequences of the ankyrin-repeat proteins, set forth in SEQ ID NO:41, or SEQ ID NO: 43.

Using conventional techniques, antisense molecules, double stranded RNA, triple helix DNA and ribozymes, directed to an appropriate nucleotide sequence of a genetic modifier, may be created. Modifications of gene expression can be obtained by designing antisense molecules, DNA, or RNA, to the control regions of the genes listed in Table 1, i.e. the promoters, enhancers, and introns. Oligonucleotides derived from the transcription initiation site, e.g., between positions -10 and +10 from the transcription start site, are preferred. Similarly, inhibition can be achieved using "triple helix" base-pairing methodology. Triple helix pairing is useful because it causes inhibition of the ability of the double helix to open sufficiently for the binding of polymerases, transcription factors, or regulatory molecules. Recent therapeutic advances using triplex DNA have been described in the literature (Gee, J.E. et al. (1994) In: Huber, B.E. and B. I. Carr, *Molecular and Immunologic Approaches*, Futura Publishing Co., Mt. Kisco, N.Y.). The antisense molecules may also be designed to block translation of mRNA by preventing the transcript from binding to ribosomes.

Ribozymes, enzymatic RNA molecules, may also be used to catalyze the specific cleavage of RNA. The mechanism of ribozyme action involves sequence-specific hybridization of the ribozyme molecule to complementary target RNA, followed by

endonucleolytic cleavage. Examples which may be used include engineered hammerhead motif ribozyme molecules that can specifically and efficiently catalyze endonucleolytic cleavage of sequences encoding the gene products of Table 1.

5           Specific ribozyme cleavage sites within any potential RNA target are initially identified by scanning the target molecule for ribozyme cleavage sites which include the following sequences: GUA, GUU and GUC. Once identified, short RNA sequences of between 15 and 20 ribonucleotides corresponding to the region of the target gene containing the cleavage site may be evaluated for secondary structural features which  
10   may render the oligonucleotide inoperable. The suitability of candidate targets may also be evaluated by testing accessibility to hybridization with complementary oligonucleotides using ribonuclease protection assays.

          Antisense molecules and ribozymes of the invention may be prepared by any  
15   method known in the art for the synthesis of nucleic acid molecules. These include techniques for chemically synthesizing oligonucleotides such as solid phase phosphoramidite chemical synthesis. Alternatively, RNA molecules may be generated by in vitro and in vivo transcription of DNA sequences encoding the genes of Table 1. Such DNA sequences may be incorporated into a wide variety of vectors with suitable RNA  
20   polymerase promoters such as T7 or SP6. Alternatively, these cDNA constructs that synthesize antisense RNA constitutively or inducibly can be introduced into cell lines, cells, or tissues.

          Vectors may be introduced into cells or tissues by many available means, and may  
25   be used in vivo, in vitro or ex vivo. For ex vivo therapy, vectors may be introduced into stem cells taken from the patient and clonally propagated for autologous transplant back into that same patient. Delivery by transfection and by liposome injections may be achieved using methods which are well known in the art.



Gene specific inhibition of gene expression may also be achieved using conventional double stranded RNA technologies. A description of such technology may be found in WO 99/32619 which is hereby incorporated by reference in its entirety.

5 Still further, such molecules may be used as components of diagnostic methods and kits whereby the presence of an allele causing diseases associated with abnormalities in the APP pathway and/or Alzheimer's Disease may be detected.

10 Other objects, features, advantages and aspects of the present invention will become apparent to those of skill from the following description. It should be understood, however, that the following description and the specific examples, while indicating preferred embodiments of the invention, are given by way of illustration only. Various changes and modifications within the spirit and scope of the disclosed invention will become readily apparent to those skilled in the art from reading the following  
15 description and from reading the other parts of the present disclosure.

### EXAMPLES

The following procedures are performed to conduct the examples:

#### 20 Transgenic Flies

Methods for the creation of transgenic *Drosophila melanogaster* flies are well known to one of skill in the art. Any conventional method can be employed, for example, the basic laboratory techniques that are involved in the creation of the flies of  
25 the present invention are described in Spradling, above. As contemplated herein, transgenic flies may be created by direct fusion of DNA sequences of interest with expression control sequences as described below. For example, transformed strains are generated using the constructs discussed above according to conventional methods. Several independent insertions may be obtained for the constructs, UAS-Abeta40, UAS-  
30 Abeta42, UAS-C99wt, UAS-C99V717I (London mutation) and pGMR-Abeta42.

Fly stocks

Gal4 lines that may be used to drive expression of the transgenes in the transgenic flies of the present invention include, but are not limited to, apterous-Gal4 and 7B-Gal4. Descriptions of the Gal4 lines mentioned and notes about their specific expression patterns can be found in Flybase (<http://flybase.bio.indiana.edu>). New transgenic strains generated in house include strains carrying UAS Abeta<sub>40</sub> and Abeta<sub>42</sub>, UAS C99 wild type and UAS C99 London (carrying the London FAD Alzheimer's-associated mutation) and GMR Abeta<sub>42</sub> transgenes.

The *yw; BcElp/CyOHop* strain, expressing transposase, and the strains *yw; Gla/SM6a* and *yw; Dr/TM3 Sb Ser* were obtained from R. Padgett, Waksman Institute, Rutgers University. *w<sup>1118</sup>* flies and *GMR-GAL4* flies were from the Bloomington stock center. The *pGMR-1* strain is a publicly available stock and was obtained from G. Rubin's lab at UC Berkeley.

DNA constructs and molecular techniques

A DNA fragment coding for the A $\beta$  42 peptide and fused to the human pre-proenkephalin signal peptide is PCR amplified and cloned into the Bgl II site of the *Drosophila* eye-specific P element transformation vector, pGMR (Hay et al., 1994 Development 120:2121-2129) and the insert is sequenced by automated fluorescence sequencing (ACGT Inc.). The human pre-proenkephalin signal peptide has been shown to successfully drive secretion of A $\beta$  42 from transfected mammalian cells. GMR is composed of five tandem copies of a response element derived from the *rhodopsin-1* gene promoter, a binding site for the eye-specific transcription factor GLASS (Ellis et al., 25 Development 119(3):855-65 (1993). Thus, Abeta expression is driven in the pattern of the GLASS transcriptional activator in the eye. The above DNA fragment is subsequently cloned into a P-element containing vector that facilitates the insertion of the transgene into the *Drosophila* genome. All molecular manipulations are done according to standard protocols. (See, for example, Sambrook, Fritsch and Maniatis, "Molecular Cloning A Laboratory Manual, 2<sup>nd</sup> Edition, Cold Spring Harbor Laboratory Press, 1989).

A DNA fragment coding for the C99 peptide and fused to the human pre-proenkephalin signal peptide is PCR amplified and cloned into the pUAST transformation vector as described in Brand and Perimmon.

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The constructs UAS-Abeta40, UAS-Abeta42, UAS-C99wt and UAS-C99V7171 contain the pre-proenkephaline gene signal peptide followed by fragments of human Abeta (40 or 42) or C99 (wild type or with the London mutation). The human fragments are cloned into the pUAST vector as described in Brand and Perrimon, above. Cloning  
 10 into this vector places the UAS sequence upstream of the transcribed region of the inserted gene and also allows integration into the fly genome through P-element recombination.

#### 15 Genetic crosses, analysis and visualization of phenotypes

Flies are crossed according to conventional methods except that all crosses are kept at 29 °C for maximal expression of phenotypes. In the binary Gal4 expression system, this temperature maximizes activity of the Gal4 protein. In the case of pGMR-Abeta42, it is observed that the phenotype is stronger at 29 °C, so these flies are kept at  
 20 this temperature as well.

#### Western analysis

Ectopic gene expression can be assayed by performing Western analysis  
 25 according to conventional methods. Antibodies that may be used include the human 6E10 monoclonal antibody raised against the beta amyloid portion of the *APP* gene and which also recognizes the C99 portion of *APP* (Senetek PLC, Napa, CA).

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### Western Protocol

To detect expression of the A $\beta$  42 peptide, flies of genotypes *K18.1/K18.1*, *K18.3/K18.3*, *K18.1/K18.1;K18.3/K18.3*, *KJ103/TM3Sb Ser*, *KJ103/KJ103*, *KJ54/CyO;KJ54/TM2 Ubx* and *pGMR-1* (flies carrying pGMR vector without insert) are reared at 29°C. 80-90 *Drosophila* heads from each of the above strains are collected, placed in an eppendorf tube on dry ice containing 100 $\mu$ l of 2% SDS, 30% sucrose, 0.718 M Bistris, 0.318 M and Bicine, with "Complete" protease inhibitors (Boehringer Mannheim) and are ground using a mechanical homogenizer. Samples are heated for 5 min at 95°C, spun down for 5 min at 12, 000 rpm, and supernatants are transferred into a fresh eppendorf tube. 5%  $\beta$ -mercaptoethanol and 0.01% bromphenol blue are added and samples are boiled prior to loading. Approximately 200ng of total protein extract is loaded for each sample, on a 15% Tricine/Tris SDS PAGE gel containing 8M Urea. The A $\beta$  1-42 peptide control is human  $\beta$ -amyloid [1-42] (BIOSOURCE International, # 03-111). Samples are run at 40V in the stacking gel, and at 120V in the separating gel. Samples are transferred to PVDF membranes (BIO-RAD , # 162-0174) for 1 hr @ 100V, and the membranes are subsequently boiled in PBS for 3 min. Antibody hybridization is as follows: the primary Ab 6E10 (SENETECK PLC, # 300-02), which recognizes the first 19 amino acids of the A $\beta$  peptide, is used for probing (at a concentration of 1:2000) in 5% non-fat milk, 1X PBS containing 0.1% Tween 20, for 90 min @ RT. Samples are washed 3 times for 5 min., 15 min. and 15 min. each, in 1X PBS-0.1% Tween-20. The secondary Ab is anti-mouse-HRP (Amersham Pharmacia Biotech, # NA 931) and is used at 1:2000 in 5% non-fat milk, 1X PBS containing 0.1% Tween 20, for 90 min at RT. Samples are washed 3 times for 5 min., 15 min. and 15 min. each, in 1X PBS-0.1% Tween-20. ECL (ECL Western Blotting Detection Reagents, Amersham Pharmacia Biotech, # RPN 2209) is used for detection.

### Histology

Plastic sections of fly heads are performed according to conventional methods, for example, as according to the protocols described in : *Drosophila Protocols*, page 236. Eds. W. Sullivan, M. Ashburner, S. Hawley, CSHL Press 2000

### Cryosections

Adult eyes are cryosectioned according to Wolff, in *Drosophila Protocols*, CSHL Press, 2000, sections 13.1 and 13.2. The primary antibody is the monoclonal 6E10 (Senetek), recognizing the human A $\beta$  42 peptide, used at a dilution of 1:3000. The detection system is the Vectastain ABC Kit (with biotinylated anti-mouse IgG secondary, and Horseradish peroxidase H) (Vector Laboratories). The following modifications are made to the protocol by Wolff: prior to incubation with the 6E10 primary antibody, cryosections are blocked in blocking solution containing normal horse serum, according to the Vectastain ABC Kit protocol. Incubation with the secondary (preadsorbed with pGMR-1 eye tissue) is done in PBS/1%BSA containing 1-2% normal horse serum, also according to the Vectastain ABC Kit protocol. The procedure for the ABC Kit is followed; incubations with the ABC reagent are done in PBS/0.1% saponin, followed by 4X 10 min. washes in PBS/0.1% saponin. Sections are then incubated in 0.5 ml per slide of the Horseradish Peroxidase H substrate solution, 400ug/ml 3,3'-diaminobenzidine (DAB), 0.006% H<sub>2</sub>O<sub>2</sub> in PBS/0.1% saponin, and the reaction is stopped after 3 min. with 0.02% sodium azide in PBS. Sections are rinsed several times in PBS and dehydrated through an ethanol series before mounting in DPX (Fluka).

### RNA profile characterization for compound screening

RNA profiles may be assayed according to known methodology, including use of traditional Northern blot analysis as well as microarray chip technology (Incyte Pharmaceuticals, Palo Alto, CA; Affymetrix, Santa Clara, CA).

## **EXAMPLE 1**

### The Rough Eye Phenotype Induced by Ectopic Expression of A $\beta$ 42

In order to elucidate the largely unknown pathways and mechanism(s) by which

A $\beta$  42 causes neurodegeneration, the *Drosophila* eye, a neural tissue, is used as a model. In an effort to mimic the disease-specific A $\beta$  42 overexpression, transgenic flies whose genome comprises the GMR-A $\beta$ 42 amyloid transgene are created using the GMR fusion expression system disclosed above in order to ectopically express the transgene in the developing *Drosophila* eye.

#### I. A $\beta$ 42 overexpression causes rough eye phenotypes

In order to express the A $\beta$  42 peptide in the *Drosophila* eye, the A $\beta$  42 sequence is cloned into the pGMR vector. The pGMR (Glass Multimer Reporter) vector contains a pentamer of truncated binding sites for the Glass transcription factor. Glass is expressed widely during eye development, starting in the eye discs, the precursors of adult *Drosophila* eyes, where it is detected in differentiating photoreceptor neurons. It continues being expressed specifically in the eye during pupal and adult development (Moses et al, 1989 Nature 340 (6234):531-536; Moses and Rubin, 1991 Genes Dev. 5:583-593). GMR-element expression in ~2 week-old flies is examined using a reporter gene and good expression detected, suggesting that GMR element is active well into adulthood. Thus, GMR-regulated expression is directed to the eye tissue throughout the development of the eye, as well as during adulthood, making it a suitable system for expression of A $\beta$  42.

Two independent transgenic lines are originally established with the pGMR-A $\beta$  42 construct, *K18.1* and *K18.3*. In addition, another transgenic line, *pGMR-1*, expressing the same vector without an insert, is examined as a negative control. Control flies with the *pGMR-1* transgene and flies carrying one copy of either the *K18.3* or the *K18.1* transgene do not show a rough eye. Similarly, flies carrying one copy each of both of the above transgenes (*K18.3* and *K18.1*) or two copies of the *K18.1* transgene also have wild type eyes. In contrast, flies with two copies of *K18.3* have a mild rough eye phenotype; examination of fly eyes under light microscopy indicate that ectopic overexpression of A $\beta$  42 disrupts the regular trapezoidal arrangement of the photoreceptor cells of the ommatidia (identical single units, forming the *Drosophila* compound eye. The above observations suggest that there might be a dose response of the rough eye phenotype to

the copy number of transgenes present in the fly genome. To further examine this hypothesis, the number of transgenes is increased to three (2 copies of *K18.1* with one copy of *K18.3* or one copy of *K18.1* with two copies of *K18.3*). These strains also showed rough eyes. Finally, when four copies of the transgene were present (2 copies of *K18.1* with 2 copies of *K18.3*), flies showed a much more severe rough eye phenotype confirming the dose response hypothesis. The penetrance of the rough eye phenotypes is 100% in all genetic combinations. It must also be noted that a more severe phenotype is observed when flies are reared at 29°C. A temperature requirement for expressivity of eye phenotypes has been described previously (Karim and Rubin, 1998 Development 125(1):1-9) and may be specific for the eye, even though it is not restricted to GMR-containing expression systems. Such dependence could be attributed to higher transcriptional and/or metabolic rates, or altered protein conformation at the higher temperature.

II. A $\beta$  42 transgenics display rough eye phenotypes, the severity of which depends on transgene copy number.

It is well established that the expression level of transgenes in *Drosophila* depends on the chromosomal location of the specific insertions, a phenomenon known as “position effect” (Kellum, R. and Schedl, P. (1991). *Cell*. 64: 941-50). It is possible then, by generating additional independent insertions with the same transgene, to recover transgenic lines that express different levels of the transgenic protein. Thus, it might be possible to isolate transgenic lines that would express the A $\beta$  42 transgene at a high enough level to cause a phenotype at a lower temperature (25°C), thus reflecting more physiological conditions. To test this hypothesis, new insertions of the pGMR-A $\beta$  42 transgene in the fly genome are generated, using “P-element hopping” (Robertson, H.M. et al. (1988). *Genetics* 118: 461-470).

A total of 19 independent lines of the pGMR-A $\beta$  42 construct in new chromosomal locations are established. The new strains are judged as carrying new insertions based on the chromosomal linkage or homozygous lethal condition of the

transgene, as well as by differences in eye color (caused by differential expression levels of the *white* gene, used as a transformation marker). Young larvae of the above new strains are subsequently raised at 29°C until eclosion and examined for the presence of an eye phenotype. Of the 19 new lines, 7 lines, or 38%, show a rough eye phenotype. The strains that display a rough eye phenotype are subsequently raised at 25°C and scored for an eye phenotype.

The new transgenic lines show varying degrees of phenotypic severity, some of them displaying a more severe phenotype than what was originally observed in the *K18.1* and *K18.3* line. One such example is the *KJ.103* line, in which one copy of the transgene renders the adult eyes mildly rough, characterized by the presence of interspersed darker “spots” (corresponding to deeper-red pigmented ommatidia) on the ventral side of the eye, while two copies of the transgene cause extensive disorganization of photoreceptors. More importantly, this specific line displays the rough eye phenotype even when the flies are raised at 25°C. When *KJ.103* flies are raised at 29°C, the severity of phenotype caused by either one or two copies of the transgene is increased dramatically.

In summary, rough eye phenotypes caused by the A $\beta$  42 peptide show a range of severity. The very mild lines typically display numerous dark/black “spots” on the ventral side of the eye, while mild lines have a more rough, disorganized appearance covering the ventral portion of the eye. Moderate lines show greater roughness over the entire eye, while in more severe lines the entire eye seems to have lost/fused many of the ommatidia and interommatidial bristles, and the entire eye has a smooth, glossy appearance. Interestingly, the size of the eye is only moderately affected in flies with the highest level of the A $\beta$  42 expression (strain *KJ54*). This is consistent with observations in flies expressing human  $\alpha$ -synuclein (Feany, M.B. and Bender, W.W. (2000) *Nature* 404:394-398. In flies expressing poly-glutamine expanded human huntingtin, a very slight reduction of eye size is observed, in the strongest-expressing transgenic lines (Warrick, J.M., et al (1998). *Cell* 93: 939-949). The above results suggest that neurodegeneration induced by over-expression of human disease genes differs from the phenotypes caused by overexpression of genes acting in apoptotic pathways (Grether, M.



E. et al. (1995). *Genes Dev.* 9, 1694-1708), in which the size of the eye is primarily affected.

Based on the above results, it is hypothesized that the severity of the rough eye phenotype depends on the amount of Abeta protein present. As a consequence of this hypothesis, it should be expected that the KJ.103 transgene displays a higher level of protein expression than the K18.3 transgene (see below).

### 10 III. Expressivity of the rough eye phenotype correlates with A $\beta$ 42 protein levels

To determine if the severity of the rough eye phenotype correlates with expression levels of the A $\beta$  42 peptide, Western blot analysis of protein extracts from *Drosophila* heads are performed (strains used are described in methods above). Results indicate that animals with two copies of the transgene have roughly twice the amount of A $\beta$  42 peptide than animals with one transgene copy. Interestingly, even though flies with two copies of *K18.1* express the A $\beta$  42 peptide in detectable quantities, they have no visible adult eye phenotype. Flies with two copies of the higher-expressing *K18.3* transgene, expressing overall larger quantities of A $\beta$  42 peptide do show the rough eye phenotype. This is also true for flies expressing two copies of the K18.1 and two copies of the K18.3 transgenes. Flies expressing only one copy of the KJ103 transgene have roughly equal amounts of protein as flies expressing two copies of the K18.3 transgene, confirming the hypothesis that the KJ103 transgene shows higher levels of relative protein expression.

The above results indicate that there is a requirement for a certain level of A $\beta$ 42 protein in order to generate a visible phenotype. It is still possible that lower amounts of A $\beta$  42 expression cause minor disruptions that would only be visible at the ultrastructural level. To test this, thin sections (1.5 $\mu$ m) from adult fly heads are examined. These data indicate that, compared to eyes from a fly carrying the empty pGMR vector, in which the toluidine-blue staining photoreceptors are regularly arrayed, flies carrying one copy of the moderately expressing K18.3 transgene have small abnormalities-some

photoreceptors are missing, blue-staining masses are forming around the ommatidia and some gaps are appearing in the tissue. These eyes appear normal macroscopically.

Sections from eyes expressing two copies of the K18.3 transgene, in agreement  
 5 with observations at the macroscopic level, display variable disorganization. As the  
 phenotype gets worse, the concentration of dense, staining masses around the ommatidia  
 increases, as do the gaps in the tissue. The ommatidia look smaller and are missing  
 photoreceptors. Two copies of the higher expressing KJ103 transgene show a phenotype  
 similar in severity. Finally, eyes from *Drosophila* expressing four copies of the strong  
 10 expressing KJ54 transgene show an almost complete loss of photoreceptors.  
 Additionally, these eyes show an abundance of dense, staining masses and of tissue gaps.  
 Even though it is not clear at this point whether the dense, staining masses that surround  
 the ommatidia are abnormal/dying cells or whether they contain aggregating Abeta  
 peptide, it is clear that their accumulation is coincident with observed overall eye  
 15 degeneration.

In order to visualize the expression of beta-amyloid on the eye tissue, sections of  
 A $\beta$  expressing eyes are stained with an antibody recognizing the human A $\beta$  peptide.  
 Transverse sections of eye tissue show a punctate staining that is absent in controls. It is  
 20 hypothesized that this punctate staining corresponds to small aggregates/deposits of beta  
 amyloid. Cellular localization of this staining as well as the exact nature of the  
 aggregate/deposit, using known A $\beta$  staining dyes is under investigation.

In summary, it is disclosed herein that introduction of more copies of the A $\beta$  42  
 25 transgene in the *Drosophila* eye, reflected by increased levels of A $\beta$  protein, has an  
 additive affect on the rough eye phenotype. It is possible that a certain concentration of  
 the A $\beta$  42 peptide is needed to affect its aggregation/conformation state. Alternatively,  
 saturating levels of the peptide might be needed for manifestation of the toxic effect. The  
 fact that A $\beta$  exerts neurotoxic effects in several signaling pathways, (intracellular calcium  
 30 levels, oxidative stress, inflammatory response, muscarinic and nicotinic receptor  
 signaling, reviewed in Fraser, S.P., et al (1997). *Trends Neurosci.* 20: 67-72; Mattson,

M.P. (1997). *Physiol. Rev.* 77: 1081-1132; and Coughlan, C.M. and Breen, K.C. (2000). *Pharmacol. and Ther.* 86: 111-144; Hellström-Lindahl and Court, 2000 *Behav Brain Res.* 113 (1-2):159-168), might indicate the need for saturating levels in order to cause disruptions. It is clear however, that expressing moderate amounts of the peptide seem to  
 5 have no consequence for the structure of the adult eye at the gross morphological level.

#### IV. Rough eye phenotype induced by A $\beta$ 42 peptide worsens with age

10 It is well established that in Alzheimer's patients, chronic accumulation of A $\beta$  peptide leads to initial manifestation of the disease and to progressive worsening of the symptoms. In order to test whether one could mimic this aspect of the disease in the *Drosophila* model, the degree of roughness of the eye phenotype in aged flies is recorded.

15 Two strains of flies, expressing pGMR1 (as a negative control) and K18.3 are examined. K18.3 flies are used because in this transgenic strain there is a range of phenotypic severity and thus it is easier to record changes. Flies from the two strains are raised at 25°C and 0-2days after eclosion they are transferred to 29°C, to induce higher expression of the transgene. Flies are scored for eye phenotype approximately every  
 20 week, for a total of one month, thereafter. The K18.3 flies are classified into three different groups (moderate, mild, intermediate), according to the observed severity of the eye phenotype. As mentioned previously, pGMR1 expressing flies did not show any eye phenotype.

25 Data indicates a shift in the phenotypic severity of the Abeta expressing flies as they age: when flies first eclose, no eyes with an intermediate phenotype are observed, whereas 15% of the population at seven days has an intermediate phenotype. Also by seven days, all of the progeny show a degree of rough eye phenotype, whereas 42% do not show any phenotype upon eclosion. By 32 days, even though a large number of flies  
 30 have died, the overall ratio of flies with mild versus intermediate phenotype is not

significantly changed, suggesting that the maximum effect of Abeta expression has been reached.

The *Drosophila* model disclosed herein appears to be mimicking the progressive and age-associated worsening of the Alzheimer's disease symptoms, an important aspect of the disease. The observed increase in the severity of the eye phenotype as flies age could be attributed to increased sensitivity of neuronal cells to the levels of A $\beta$  peptide. Indeed, as mentioned above, A $\beta$  peptide is being produced throughout the adult stage of *Drosophila*. It is thus possible that increased levels of A $\beta$  cannot be effectively turned over, resulting in accumulation of the peptide in the *Drosophila* cells. Alternatively, it is possible that aged cells are more vulnerable to the presence of A $\beta$  peptide.

#### V. The rough eye phenotype and the degree of apoptotic cells in larval eye imaginal discs and adult eyes

As mentioned earlier, the A $\beta$  42 peptide has known toxic effects and it is suggested that it plays a role in apoptosis. Based on this, third instar larval eye imaginal discs, the precursors of the adult eye, are examined for evidence of apoptosis, or programmed cell death. Dissected eye imaginal discs from *K18.1/K18.1*; *K18.3/K18.3* larvae, raised at 29°C, are stained with acridine orange according to conventional methods, which causes fluorescence of apoptotic cells. As controls, the following strains, none of which shows any eye abnormalities, are used: *w<sup>1118</sup>* (a wild-type control) and *pGMR-1* (carrying the "empty" pGMR vector) grown at 29°C and *GMR-GAL4* (expressing Gal4 under the control of the GMR element), raised at 18°C.

Results indicate that little or no cell death is seen in the wild-type control, *w<sup>1118</sup>*. In contrast, some amount of cell death can be detected in the *K18.1/K18.1*; *K18.3/K18.3* line. When the controls that carry the pGMR vector but do not display any eye phenotype (*pGMR-1* and *GMR-GAL4*), are examined, some cell death is also observed, comparable in extent to that observed in the experimental flies, *K18.1/K18.1*;

*K18.3/K18.3*. Therefore, it seems likely that a certain amount of cell death is tolerated during eye development and does not cause any adult eye defects, at least at the gross morphological level. In addition, it is suggested herein that if apoptosis has any involvement in the generation of the rough eye phenotype, it is not manifested during the early development of the eye.

To test whether the observed rough eye phenotype is caused by apoptosis during the adult stages of *Drosophila*, the apoptosis inhibitor DIAP1 is co-expressed in the *Drosophila* eye. Co-expression of DIAP1 in eyes expressing Abeta would be expected to suppress, at least partially, the rough eye phenotype (data not shown). Since no suppression with two different DIAP-expressing strains is observed, it may be that the observed rough eye phenotype is not caused by ectopically induced apoptotic cell death. The same results were obtained when the antiapoptotic baculoviral P35 gene was used. These results suggest that the effects caused by the A $\beta$  42 peptide in the *Drosophila* eye might be mediated by cellular pathways that do not result in apoptosis.

The actions of A $\beta$  42 are quite complex and could affect other proteins known to be factors in AD development. It has been shown that PS 1 and PS 2 co-immunoprecipitate with APP (Xia et al., 1997 PNAS USA 94 (15):8208-13) and that A $\beta$  42 can directly bind PS 2 in vitro (Czech et al., 1999 Society for Neuroscience 25:641.1). It is interesting to note that overexpression of wild type and mutant PS forms also results in enhanced susceptibility to apoptosis in several experimental systems, including the *Drosophila* eye (Ye, Y. and Fortini, M. (1999). *J. Cell Biol.* 146: 1351-1364). In these studies, it is suggested that *Drosophila presenilin (Dps)* exerts a dominant negative effect when expressed at high levels. It is unclear how *Dps* causes apoptosis of cells, but the mechanism could involve the dysregulation of the Notch and/or Wnt developmental signaling pathways (reviewed in Anderton et al., 2000 Mol. Med. Today 6:54-59). It is unclear whether A $\beta$  42 overexpression in the system disclosed herein could be affecting *Dps* function by possibly interfering with one or more of these signaling pathways. Interestingly, overexpression of A $\beta$  40 or A $\beta$  42 enhances a *Dps* (*Drosophila presenilin*)

induced phenotype in the same tissue (data not shown), suggesting involvement of the two proteins in the same pathway.

## EXAMPLE 2

### Concave Wing Phenotype Induced by Ectopic Expression of C99

Transgenic *Drosophila* that carry a copy of pUAS-C99 (either wild type or with the London mutation ) and a copy of apterous-Gal4 are created using standard methods and as discussed above. Data indicate that these flies exhibit a malformation of their wings in that the wing blade is curved in a concave manner. These effects are confirmed with multiple independent insertions of the C99 transgene. Western analysis confirms expression of this transgene. Protein extracts from whole larvae expressing the C99 (either wild type or with the London mutation) under the control of daughterless-Gal4 (a ubiquitously expressed Gal4 driver) show a protein band of the expected size for C99, which immunoreacts with the 6E10 antibody (raised against the first 16 amino-acids of C99). Data exists that when the portion of the human *APP* gene referred to as C100 was inserted into the genome of *Drosophila* and expressed in the wing disc, it did not generate any visible phenotype (Fossgreen et al., PNAS 95:13703-13708 (1998)). In contrast, data reported herein indicate that flies transgenic with the equivalent C100 region of human APP (called here C99), fused to a different signal peptide, display a wing malformation.

## EXAMPLE 3

### Cognitive Defects Induced by Ectopic Expression of C99

Transgenic *Drosophila* that carry a copy of UAS-C99 (either wild type or with the London mutation) and a copy of 7B-Gal4 (which allows expression in the mushroom body of the brain) are created using standard techniques. Cognitive defects of these flies can be examined by conducting olfactory, locomotion or learning and memory assays

according to conventional methods. For example, altered locomotory behavior is observed in the above flies, tested using the "dark reactivity" set-up, described by Benzer, S. *PNAS* 58:1112-1119 (1967). Specifically, flies containing a copy of UAS-C99 and a copy of 7B-Gal4 do not respond to mechanical agitation as well as wild type flies, walking less quickly than wild type flies after being tapped to the bottom of the assay apparatus. The "dark reactivity" test for locomotion is also described in "Behaviour, Learning and Memory" In : *Drosophila, A Practical Approach*. Ed. D.B. Roberts (1998) Oxford University Press Inc. New York page 273.

#### EXAMPLE 4

##### Genes that Modify *Drosophila* Phenotypes as Targets for Alzheimer Disease Therapeutics

As disclosed in detail below, genetic screens were set up in order to identify genetic modifiers of the concave wing phenotype described in Example 2. Candidate modifiers tested included known modifiers of two *Drosophila* phenotypes induced by ectopic expression of *Drosophila* presenilin (Dps) in the wing and scutellum (G. Boulianne, Hospital for Sick Children, Toronto, Canada, personal communication), as well as mutations in other candidate genes. Based on the recent discovery of a chromosome 10 AD gene "hot spot", chromosomal mapping of the human homologs of the above mentioned *Drosophila* genetic modifiers was performed. Data disclosed below indicate that the human homologs of several of the genetic modifiers disclosed herein are also located on chromosome 10 and it is contemplated herein that these genes are relevant targets for the development of pharmaceuticals useful for the treatment of Alzheimer's Disease as well as other conditions associated with errors in the regulation of the APP pathway.

A total of 93 mutations were screened in order to identify genetic modifiers of the concave wing phenotype induced by ectopic expression of C99 in the wing of *Drosophila*. The screen is based on measuring the change in the penetrance of the wing phenotype when the external mutation is present. More specifically, the number of flies with mutant wings compared to the number of flies with wild type wings are counted in the experimental group (flies expressing both C99 and mutation being tested) and the control group (flies expressing only C99). The significance of the change in penetrance of the mutant phenotype is evaluated by measuring the P value by a T test, in the above mentioned four groups. Mutations were considered to significantly modify the C99 phenotype when  $P < 0.05$ .

A list of genetic modifiers that affect the C99-overexpression phenotype and the *Drosophila* genes associated with these genetic modifiers are provided in Table 1.

All of the mutations identified as modifiers of presenilin and C99 overexpression phenotypes were insertional mutations (mediated by insertion into the *Drosophila* genome of the P-retroviral like transposable element). The exact chromosomal location of each of these insertions has been previously determined (*Drosophila* Genome Project BDGP, <http://www.fruitfly.org>). In order to identify the transcript(s) affected by each of these insertions, we scanned a 10kB genomic area to the right and to the left of each insertion for known or predicted *Drosophila* transcripts. The following criteria were adopted for selection of the most likely transcript affected by a given insertion:

a) distance of transcripts from the site of insertion, and b) orientation of a transcript relative to the insertion.

If a genomic area contained more than one candidate transcripts with the same orientation as the insertion, all those closest to the insertion were selected for further analysis. The translated protein sequences of the *Drosophila* transcripts from the above analysis form "Set A".



The presence of human homologs of the above *Drosophila* proteins (in "Set A") in an AD-linked area of human chromosome 10 was examined. Two candidate regions around Sequence Tagged Site (STS) markers on human chromosome 10 have been identified (Bertram *et al.*, Ertekin-Taner *et al.*, Myers *et al.*, *Science* 290, 2302-2305, 2000) by linkage analysis. We mapped STS marker sequences used in these linkage analysis studies or STS sequences adjacent to these markers to the Celera genome data by blastn (Altschul *et al.*, 1997) sequence comparisons. Based on this mapping information a subset of human chromosome 10 was defined that included the two candidate regions showing significant linkage (Bertram *et al.*, 2000; Ertekin-Taner *et al.*, 2000; Myers *et al.*, 2000) and the region in between. The DNA sequence (Celera contigs) and the corresponding list of Celera protein translations were retrieved for the subset defined and put into blast format databases. The DNA sequence and the list of Celera protein translations for the above described genomic regions form "Set B" and "Set C", respectively.

A tblastn search with "Set A" against "Set B" and a blastp search with "Set A" against "Set C" were then performed. Initially tblastn and blastp hits with E-values lower than  $10^{-5}$  were selected. Then the best match of each fly protein from these searches was chosen and the corresponding fly genes/transcripts were checked for their association with genetic modifiers of the Dps and C99 phenotype. The resulting fourteen pairs of fly and human transcripts/proteins form "Set D".

The fourteen protein pairs in "Set D" were tested for stringent or putative orthology. This was accomplished by blastp comparisons to a combined database of all human and all fly Celera proteins. First, the fly proteins in "Set D" were compared to each protein in this database. Then the resulting best human matches for each of the fly proteins were again compared to the combined human/fly protein database. A human match was classified as a stringent ortholog if all of the following four criteria were fulfilled:

1. fly protein X has best match with human protein Y
2. fly protein X does not have a better match with another fly protein than with human protein Y
3. human protein Y has best match with fly protein X
- 5 4. human protein Y has no better match with another human protein than with fly protein X

If only criteria 1) and 3) are fulfilled, a human match is classified as a putative ortholog regardless of whether fly protein X had a better match with another fly protein, human protein Y had a better match with another human protein or both. All other human matches are deemed homologs. After the orthology test, candidate human genes are prioritized according to the following:

- a) human gene is homolog of fly gene affected by Drosophila genetic modifier, identified in genetic screen
- b) degree of sequence similarity of the human protein (encoded by the human gene in a) to the Drosophila protein (encoded by the Drosophila gene in a)
- c) human protein is stringent or putative ortholog of fly protein
- d) chromosomal location of the human gene with respect to STS markers, other candidate genes or known AD genes
- e) putative function of the human protein and / or the homologous Drosophila protein
- f) evidence that the predicted human gene is expressed
- g) existence of validated or predicted coding and/or non-coding SNPs in the coding region of the human gene.

To check whether the human homolog gene is expressed, the Incyte LifeSeq EST database was searched with the corresponding predicted human transcripts identified from the Celera database using blastn.

Based on these criteria, it is contemplated herein that 4 different human genes are AD related genes located on human chromosome 10. Below are listed the putative proteins, encoded by these proposed human genes.

- 5 (1) hCP50765 (EGR2) SEQ ID NO: 35
- (2) hCP41313 (homologous to the fly gene *nocA*) SEQ ID: 15, SEQ ID NO: 17 or SEQ ID NO 53
- (3) hCP33787 (ankyrin-related protein) SEQ ID NO: 41
- (4) hCP51594 (ankyrin-3) SEQ ID NO: 43

10

The Celera predicted transcript hCT15097 was manually curated to produce two putative forms of the protein hCP41313. Curation was performed by identifying ESTs corresponding to this locus by blastn searches of the Incyte LifeSeq and public EST databases and aligning the identified ESTs with the Celera predicted transcript sequence.

15 The curation produced slight changes in the C-terminal amino-acid sequence and putative additional residues at the N-terminus of the predicted amino-acid sequence. The changes in the C-terminal part of the human protein sequence lead to an improved alignment with the fly *nocA* in this region. Because of the additional residues at the N-terminus Met 64 and Met 100 in the Celera protein sequence (hCP41313) correspond to Met 114 and Met

20 150 in the translation of the complete curated *nocA* a homolog transcript sequence respectively. We have subsequently analyzed and compared cDNA sequences from the Novartis FGA cDNA collection and the Incyte cDNA collection. Based on these analyses we have cloned and sequenced a cDNA clone corresponding to the human *nocA* gene on chromosome 10. The 5' end of this cDNA clone consists of cDNA obtained

25 from proprietary Novartis clone *fga94341* and the 3' end of this clone consists of cDNA obtained from Incyte clone 242278.1 (SEQ ID NO: 52 , SEQ ID NO. 53).

EGR2 is a putative ortholog of Celera predicted fly transcript CT23724 (see Table

30 1), which corresponds to the *Drosophila stripe* gene. The fly mutation P1505 (see Table 1), which affects the *stripe* gene, modifies only the presenilin phenotype.

Human *nocA* is a putative ortholog of Celera predicted fly transcript CT14619 (see Table 1), which corresponds to the *Drosophila nocA* gene. The fly mutation EP2173 (see Table 1), which affects the *nocA* gene, modifies both the presenilin and C99 overexpression phenotypes.

EGR2 is a C2H2 type zinc finger transcription factor regulating PNS myelination. In mice it has been shown to be important for hindbrain development (Schneider-Maunoury *et al.*, *Cell* 75, 1199-1214, 1993; Swiatek & Gridley, *Genes Dev.* 7, 2071-2084, 1993). Four mutations in EGR2 have been described to be associated with inherited peripheral neuropathies (Warner *et al.*, *Nature Genet* 18, 382-384, 1998; Timmerman *et al.*, *Neurology* 52, 1827-1832, 1999).

The *nocA* human homologue is a putative transcription factor with a C2H2 type zinc finger domain. While its exact function has yet to be determined, according to data disclosed herein, it may play a significant role in the pathology of Alzheimer's Disease. The *Drosophila* protein encoded by the *nocA* gene is a transcription factor involved in the development of the embryonic brain and the adult ocellar structures.

Ankyrin-3 exists in two brain specific isoforms of 480 and 270 kDa (Kordeli *et al.*, *J Biol Chem* 270, 2352-2359, 1995). Neural-specific Ankyrin-3 polypeptides are candidates to participate in the maintenance and targeting of ion channels and cell adhesion molecules to nodes of Ranvier and axonal initial segments. Ankyrin-3 has been shown to associate with the voltage dependent sodium channel *in vitro* and to co-localize with this molecule at nodes of Ranvier, axonal initial segments, and the neuromuscular junction (Srinivasan *et al.*, *Nature* 333, 177-180, 1988; Kordeli *et al.*, *J Cell Biol* 110, 1341-1352, 1990; Kordeli & Bennett, *J Cell Biol* 114, 1243-1259, 1991; Flucher & Daniles, *Neuron* 3, 163-175, 1989).

The second human homologue of fly transcript CT18415 belongs to the family of ankyrin-related proteins (hCP33787). The corresponding gene is located 469 kbp from

insulin-degrading enzyme (IDE). In addition to ankyrin-repeats, hCP33787 contains a sterile alpha motif (SAM) domain. The SAM domain has been suggested to be involved in the regulation of developmental processes (Shultz *et al.*, *Protein Sci* 6, 249-253, 1997), has been described as mediating specific protein-protein interactions, and has been suggested to form extended polymeric structures (Thanos *et al.*, *Science* 283, 833-836). The SAM domain is included in the alignment between fly transcript CT18415 and hCP33787. We speculate that it might play a role in the aggregation of  $\beta$ -amyloid.

It has been hypothesized that  $\gamma$ -synuclein might be involved in AD (Luedeking *et al.*, *Neuroscience Letters* 261, 186-188, 1999). We postulate that  $\gamma$ -synuclein might interact with the ankyrin repeat-containing protein hCP33787. In support of this, a protein-protein interaction between synphilin, an ankyrin repeat-related protein, and  $\alpha$ -synuclein has been shown (Engelender *et al.*, *Nature Gen* 22, 110-114, 1999). It is also known that members of the synuclein family share a high degree of sequence similarity (64% sequence identity between  $\alpha$ -synuclein and  $\gamma$ -synuclein, Lavedan, *Genome Res* 8, 871-880, 1998). Since the fold of an ankyrin-repeat unit is conserved, the above arguments add support to a putative protein-protein interaction between hCP33783 or hCP51594 and  $\gamma$ -synuclein. It is of interest to note that a coding SNP (E  $\rightarrow$  K) is predicted for hCP33787 at sequence position 48, which corresponds to the start of the ankyrin-repeat region. This sequence variation could be relevant in the context of a putative  $\gamma$ -synuclein – ankyrin interaction because it involves oppositely charged amino acid side chains. The predicted SNP in the ankyrin-related protein is particularly interesting as  $\gamma$ -synuclein has a validated coding SNP (V $\rightarrow$ E) at position 110 (Ninkina *et al.*, *Hum Mol Genet* 7, 1417-1424, 1998), that codes for the exchange of a neutral by a negatively charged amino acid side chain. We postulate that these polymorphisms might be relevant to a putative interaction of  $\gamma$ -synuclein with either hCP33783 or hCP51594.